NEW SERIES VOL. 22 NO. 88 OCTOBER 1958

ANNALS OF BOTANY

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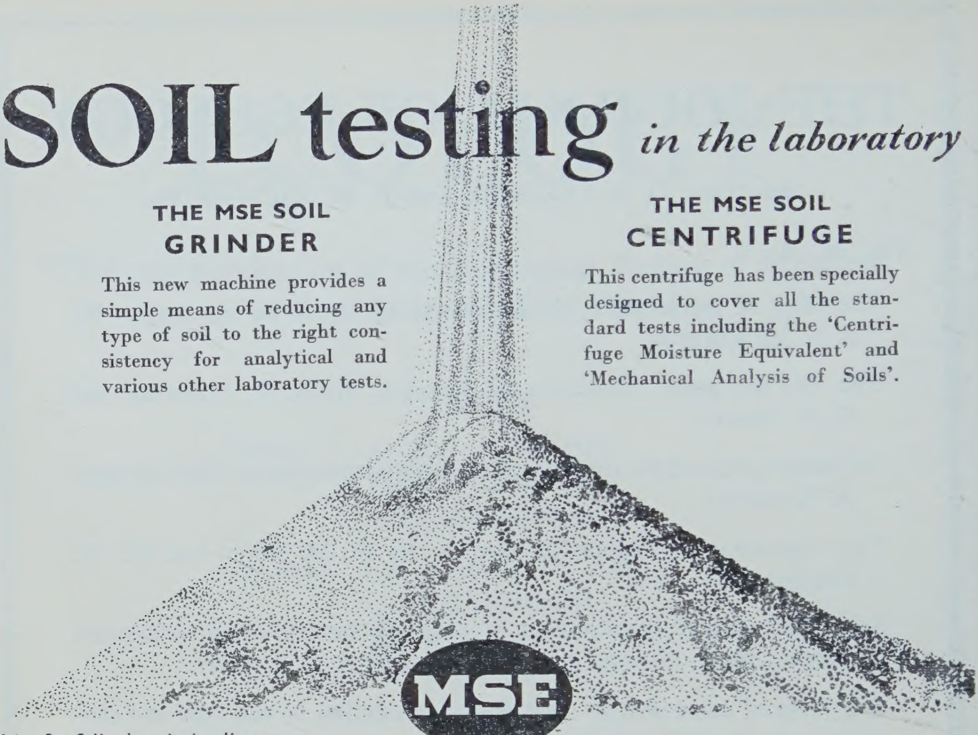

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An Apparatus for Maintaining Disks of Tissue in a Uniform Environment

BY

I. R. MACDONALD AND A. H. KNIGHT

(*Macaulay Institute for Soil Research, Craigiebuckler, Aberdeen*)

With one Figure in the Text

ABSTRACT

An apparatus is described in which tissue disks may be conveniently maintained in uniform environmental conditions. Particular attention is paid to temperature control.

INTRODUCTION

THE pioneer work of Stiles and Steward and their collaborators established the general suitability of disks of storage tissue for studies in plant metabolism. The metabolism of the plant is basically that of the individual cell and fundamental research should therefore be pursued at the cellular level. Since methods of ultra-micro analysis are not generally available, units of plant tissue which contain a sufficient number of essentially similar cells have to be chosen so as to allow the use of micro or semi-micro methods of investigation. Apart therefore from the obvious convenience of handling small disks as compared with unmanageable swollen storage roots, disks possess the additional merit of providing a ready and constant supply of uniform experimental material. Moreover, the metabolic activity of disks can be maintained over comparatively long periods. Stiles (1927), working with red-beet tissue, showed that the disks, under suitable conditions, retained their vitality for several weeks, and Turner (1938) states that carrot tissue in the form of disks retains its nature as a mass of organized cells for many weeks. These attributes facilitate the study of metabolic changes associated with ageing.

However, if the disks are to be used in experiments of prolonged duration it is essential that they be kept in uniform environmental conditions, and it is difficult to assess the value of experiments with disks unless the conditions obtaining during their pretreatment have been clearly stated. Steward (1932) has shown that potato disks are very sensitive to altered environmental conditions which cause alterations in the cell metabolism, and that any variables in the pretreatment must be rigorously controlled. His work on oxygen effects is well known but in addition he has shown that the rate of stirring exercises a profound influence on cell metabolism, the effects of overstirring being similar to those of reduced oxygen. Stiles (1927), on the other hand, working with red beet, showed that agitation was essential to keep the disks in a healthy condition; disks left to lie in distilled water rapidly lost their electrolytes, whereas the loss was practically negligible from those which were continuously shaken.

[Annals of Botany, N.S. Vol. 22, No. 88, Oct. 1958.]

The need for exact temperature control during the measurement of oxygen uptake or salt uptake by disks is now seldom ignored but less attention has been paid to the need to maintain rigorous temperature control of the disks during pretreatment. The general practice hitherto has been to maintain the disks in continuously changing water where there would obviously be some temperature latitude (Bennet-Clark and Bexon, 1943; Stiles and Dent, 1947; Rees, 1949; Audus, 1956), or in a container in which water was changed daily either with (Sutcliffe, 1952) or without (Robertson, Turner, and Wilkins, 1947; Davies and Wilkins, 1951) temperature control.

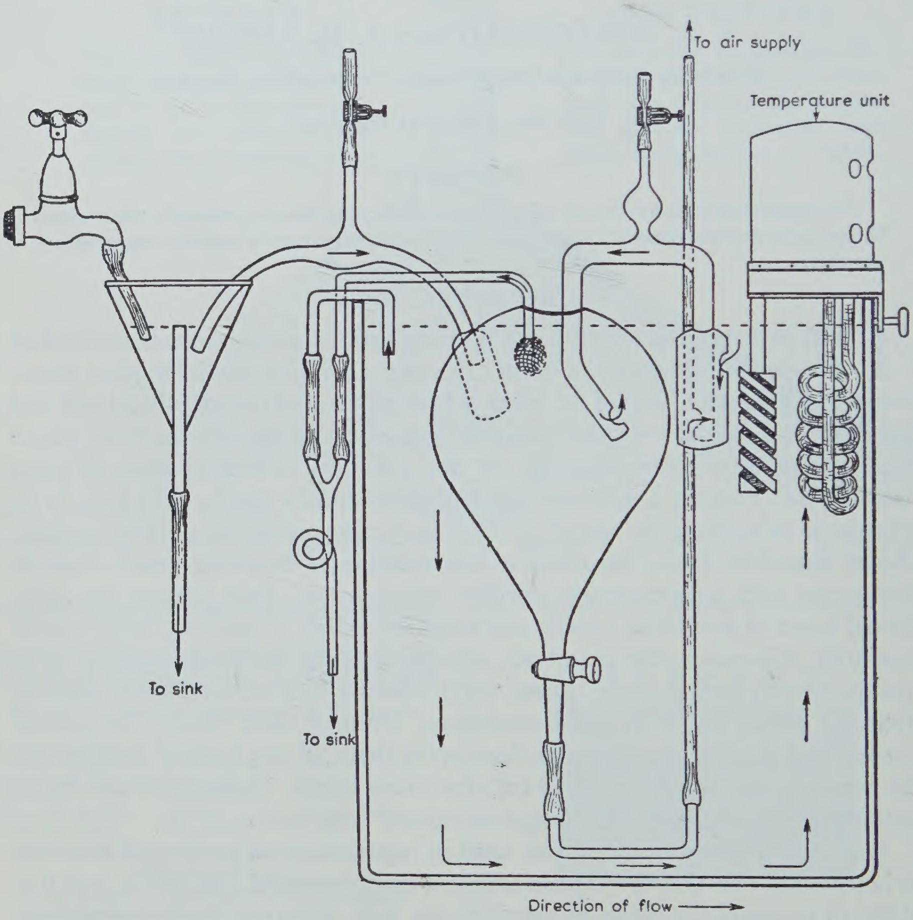

FIG. 1

Work with disks of various storage tissues shows that the conditions most conducive towards keeping the disks in a state in which their vital activities are unimpaired are such as result from constant temperature, a continuously circulating and changing water-supply, and aeration and agitation sufficient to keep the disks separate from one another. Of these, constant temperature is of particular importance. It was found when keeping disks washing in tap water without any thermostatic control that the temperature of the water could vary from 5° C. to 15° C. throughout the year. Moreover, the temperature of the water in the disk-washer was also affected by factors such as the temperature of the room, the time of day, and the rate at which water was passing into the washer. Such temperature variations were found to influence very markedly the metabolic condition of the disks and it became evident that it is essential to maintain disks at a constant temperature if comparisons are to be made between batches of disks investigated at intervals of several weeks or even between samples drawn from one batch of disks washing over a period of 1 or 2 weeks. Results obtained in respiration studies were found to be influenced by the temperature of pretreatment (MacDonald and DeKock, 1958). The apparatus shown in Fig. 1 was designed to meet the conditions enumerated above.

DESCRIPTION OF THE APPARATUS

The apparatus consists of a large glass tank of 75-litre capacity (30 × 48 × 52 cm.) in which is suspended a 5-litre Pyrex separating flask with the top cut off to give an aperture of 10 cm. diameter. The disks are circulated in this inner chamber by means of a stream of air supplied from an Austen Model MU/19 air compressor. The air-stream enters the separating flask through the stem and can be regulated to ensure that the disks are continually circulating so as to prevent the development of anaerobic conditions caused by the disks piling up on one another. The air-stream thus serves the dual purpose of aeration and circulation of water and disks. The water in the outer chamber is maintained at the required temperature and circulated by means of a Techne Tempunit having a 1 kilowatt heater. The Tempunit has a variable temperature range and can maintain the temperature of the water to within 0·05° C. It was successfully used at temperatures between 5° C. and 25° C.

The continuously changing water-supply is effected by a series of siphons. Water is withdrawn from the inner chamber by an intermittent siphon consisting of a capillary and wide-bore glass tube as previously described (DeKock, Hunter, and MacDonald, 1953). This type of siphon is necessary because air bubbles present in the water will break the flow of an ordinary siphon. To maintain the level in the inner chamber another siphon operates between it and the outer tank. This siphon must be of wide bore (about 2 cm. diameter) glass tubing and is specially designed to continue working in the presence of numerous air bubbles present in both chambers. In order to minimize the entry of air bubbles the apertures are placed laterally, the inner-chamber leg of the siphon is bent away from the main air-stream, and the outer-chamber leg is enclosed in a polythene jacket in which the water has to move downward before entering the siphon. The siphon was made with a circular dome (about 100 ml. capacity) attached to the horizontal limb in order to trap air bubbles and permit the continuous flow of water. The dome is fitted with a pinch cock through which the siphon can be primed. The level of water in the outer tank is maintained by another siphon operating between it and a reservoir. Here again it is necessary to enclose the leg of the siphon entering the main tank in a polythene jacket. Tap water runs into the reservoir which is simply an 8-in. diameter polythene filter funnel fitted with an overflow tube descending through the stem. The level of water in the inner chamber, tank, and reservoir is kept constant by the siphons and is determined by the level of the overflow tube in the reservoir.

The rate at which water enters the system is determined by the rate at which water is being withdrawn from the inner chamber by the intermittent siphon and thus the rate of replacement can be regulated by means of a screw-clip on the descending limb of the intermittent siphon. The usual rate of replacement was about 300 ml. per minute. This inflow of water was immediately brought up to the required temperature by the heater of the Tempunit and so water of the correct temperature passes into the inner chamber.

The apparatus is completely foolproof against flooding and in the event of a failure in the water-supply the level of water in the tank will not drop below the level of the intermittent siphon. A piece of plastic mesh (Tygan) tied round the intake of the intermittent siphon prevents disks from choking it. The inner chamber can accommodate over 3,000 disks 11 mm. in diameter and 1 mm. thick. Disks can be removed without interference with the apparatus which will function unattended indefinitely. If an adequate supply was available, tap water could be replaced by distilled water.

In order to keep the separating flask upright it is slung in a polythene collar made from an 8-in. polythene funnel. This collar is then suspended from wooden struts across the top of the outer tank.

Preparation of the Disks

The apparatus was designed to accommodate under controlled conditions a single batch of disks for an extended period, during which time samples of disks could be removed periodically for analysis. The disks were obtained by cutting the root (e.g. a large sugar-beet, swede, potato, &c.) on a ham-slicing machine set to thickness No. 2 on the Berkel Scale, delivering slices of approximately 1·0 mm. thickness with a standard deviation of 0·07 mm. as measured by a micrometer screw gauge. Ten slices were then fitted together and disks were struck from the slices using a sharp cork-borer of 11 mm. diameter. This was the most rapid method of obtaining disks in quantity and it has been suggested by Turner (1938) that slicing the root first with subsequent cutting of disks from the slices minimizes the bruising of the tissue. By this technique about 5,000 disks could be obtained from one large sugar-beet weighing 2–3 kg. With smaller roots such as carrot it is necessary to slice a number of roots to get a sufficient quantity of disks. Immediately after cutting the disks were given a thorough washing in tap water and transferred to the disk-washer.

ACKNOWLEDGEMENT

Grateful acknowledgement is made to Mr. A. Bleszynski for the diagram of the apparatus.

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Temperature Control and Metabolic Drifts in Ageing Disks of Storage Tissue

BY

I. R. MACDONALD AND P. C. DEKOCK

(*Macaulay Institute for Soil Research, Craigiebuckler, Aberdeen*)

With sixteen Figures in the Text

ABSTRACT

The respiratory drifts of ageing disks of sugar-beet, red beet, carrot, swede, and potato maintained at different temperatures have been determined manometrically. The general pattern of the respiratory time-drift exhibited by all tissues is that of a hyperbolic curve but its exact form is largely determined by the temperature of pretreatment, higher temperatures causing a telescoping effect. Different respiratory time-drifts obtained by earlier workers from the same tissue are shown to be explicable in terms of the temperature of pretreatment. Except in swede disks, estimations of total and reducing sugar content of the disks throughout the experimental period revealed a very considerable conversion of sucrose (or starch in potato) to reducing sugars. In sugar-beet, red beet, carrot, and potato the concentration of reducing sugar is positively correlated with the rate of respiration. The character of the respiratory time-drift in the disks is discussed in relation to substrate concentration and other possible determinants.

INTRODUCTION

ANIMAL physiologists have shown that the life of an organ may be prolonged beyond that of the composite organism by removal of the part from the whole with subsequent treatment in a suitable environment, and it has consequently been suggested that ageing is a feature of the organism rather than its separate parts. Similarly it has been shown by plant physiologists that explants of excised roots can be propagated indefinitely in culture medium—a technique which appears to eliminate senescent processes as commonly understood. On the other hand, ageing processes are enormously accelerated in disks excised from storage tissue, especially if they are maintained in moist air or a fluid medium at a high temperature. Such disks provide suitable material for an investigation of the more traditional concept of ageing, namely that it is fundamentally a problem of cellular change, the underlying mechanism of which is probably common to tissues phylogenetically remote from one another.

The present investigation was undertaken to obtain information on metabolic changes in ageing cells—information which is basic to a proper understanding of the processes broadly grouped under the term senescence. In addition it was felt that, in view of the frequent use of disks in the investigation of physiological processes such as salt absorption, a knowledge of the biochemical status of the disks might assist in the interpretation of results.

[Annals of Botany, N.S. Vol. 22, No. 88, Oct. 1958.]

Respiration, which is the most generally useful 'index of the integrated activities of the cell', and sugars form the subject material of this paper.

MATERIALS AND METHODS

Disks from the storage organs of sugar and red beet (*Beta vulgaris*), carrot (*Daucus carota*), swede (*Brassica napus*), and potato (*Solanum tuberosum*) were prepared as described by MacDonald and Knight (1958) and transferred to the disk-washing unit. The first sample of disks was generally taken 2 hours after cutting and subsequently samples were taken daily or at suitable intervals. Respiration rates, as indicated by oxygen uptake, were determined by standard Warburg techniques. Each Warburg vessel contained 10 disks in firm healthy condition immersed in 2·5 ml. distilled water. The vessels were shaken at 130 oscillations per minute through a stroke of 4 cm. At least 3 and generally 4 replicates were set up for each sample of tissue. The values were averaged and the respiration rate expressed as microlitres oxygen (μl O_2) absorbed per hour by 10 disks or as otherwise stated in the text. The 10-disk basis was found to be the most satisfactory as both the wet weight and the dry weight change with time, the former increasing and the latter decreasing. The wet weight of 10 freshly cut disks 1·1 cm. in diameter and 0·1 cm. thick was always between 1·0 g. and 1·1 g. for the five tissues used and therefore 10 disks may be conveniently regarded as weighing 1 g. Readings were taken every 30 minutes over a period of 2 hours. Respiration proceeded at a steady rate throughout the 2-hour period and good reproducibility between the replicates was obtained, usually to within 5 per cent.

Sugar was determined colorimetrically by Nelson's modification of Somogyi's method (Nelson, 1944) with the incorporation of Somogyi's new reagent (Somogyi, 1945), 5 disks being used for each estimation. Qualitative analyses of sugars were carried out by uni-directional paper chromatography using a mixture of butanol: glacial acetic acid: water as solvent and aniline hydrogen phthalate as the developing reagent.

EFFECTS OF PRETREATMENT TEMPERATURES

On respiratory measurements at 25° C. It has been remarked elsewhere (MacDonald and Knight, 1958) that earlier experiments with disks have generally been carried out using disks pretreated at room temperature and that in our experience this may mean anything between 5° C. and 15° C. depending on the rapidity with which fresh water enters the disk-washer. If, on the other hand, the disks are kept in a static tank without continuous washing, the temperature may be anything between 15° C. and 25° C. Fig. 1 shows the effect over varying periods of different temperatures of pretreatment on the respiratory rate of sugar-beet disks measured at 25° C. on successive days. Obviously the respiratory value obtained from disks, say, 3 days' old is determined by the temperature of pretreatment.

On respiratory measurements at different temperatures. That the respiratory value obtained from disks of any given age is determined not only by the temperature of pretreatment but also by the temperature of measurement is shown by the results from sugar beet disks given in Fig. 2. Disks pretreated and measured at 15° C. respire more slowly than those pretreated and measured at 25° C., whereas those pretreated at 15° C. and measured at 25° C. give (for the first week) intermediate values. Fig. 3 illustrates respiratory patterns obtained from two lots of swede disks taken from the same root and pretreated at 10° C. and 25° C. The respiration rates of both series were studied at 10° C. and 25° C. and while the results show that there is an exact parallelism between the respiration curves of disks pretreated at one temperature and estimated at different temperatures, no matter whether the temperature of pretreatment be higher or lower than that of estimation, yet it should be noted that a very different respiratory rate is obtained from disks pretreated

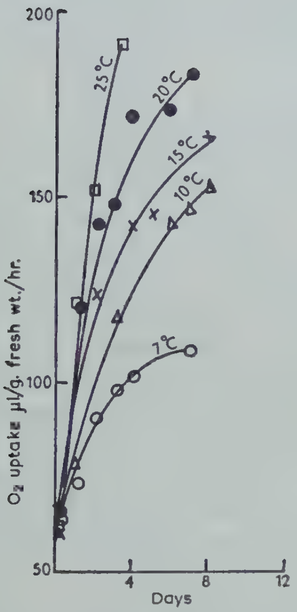

FIG. 1

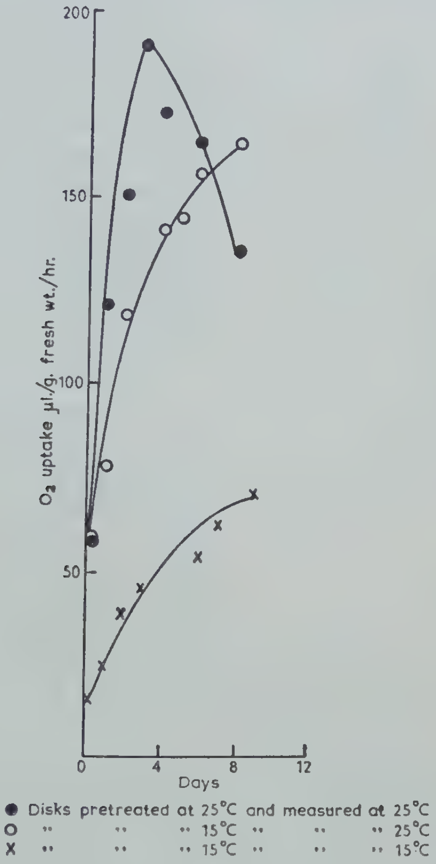

FIG. 2

FIG. 1. The effect of different pretreatment temperatures on the respiratory drift of sugar-beet disks measured at 25° C.

FIG. 2. The effect of pretreatment temperature on the respiratory drift of sugar-beet disks measured at different temperatures.

at 10° C. and measured at 25° C. in comparison with those pretreated at 10° C. and measured at 10° C., and it is important to bear this in mind (as will be shown in subsequent paper) when determining the sensitivity of the tissue to inhibitors. Transferring disks from a low to a higher temperature for respiratory estimations may result in the development of a temperature-induced cyanide-sensitive respiration.

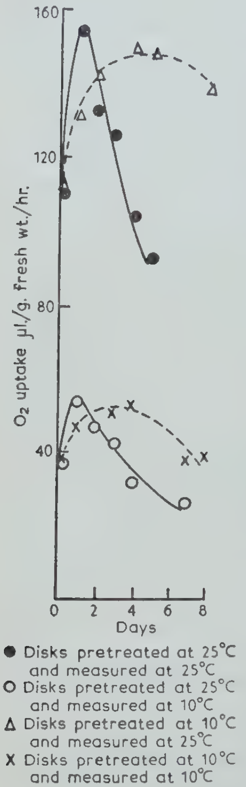

FIG. 3. The effect of pretreatment temperature on the respiratory drift of swede disks measured at different temperatures.

Clearly therefore the respiratory value obtained from disks at any particular time is a product of the temperature of pretreatment and the temperature of measurement, and the age of the tissue in days gives no indication of its physiological condition nor can this condition be assessed apart from a knowledge of these temperatures.

RESPIRATORY AND CARBOHYDRATE PATTERNS IN AGEING DISKS

Sugar-beet: Immediately after cutting, the respiration rate of disks 1 mm. thick was approximately 60 µl O_2/g. fresh weight/hour. Estimation of the respiration rate of the disks on subsequent days showed that it follows a well defined pattern, dependent on the conditions of pretreatment and measurement. The life-span of disks kept in warm water is telescoped in comparison with that of disks kept in cold water, but the pattern is essentially the same and follows that shown in Fig. 4 for disks pretreated at 10° C. and measured at 25° C. The telescoping effect of pretreatment at a higher temperature is also shown in Fig. 4 by a second curve which relates to disks pretreated and measured at 25° C.

The total sugar content of 10 disks freshly cut (i.e. 1 g. fresh tissue) was generally about 120 mg., but this value varied with individual beet and the length of storage time. The total sugar content dropped steadily as the disks aged until values as low as 2 or 3 mg. were recorded. The reducing sugar content of 10 freshly cut disks was always less than 1 mg., but after a temperature-determined interval it began to rise very rapidly, reaching a maximum value of about 40 mg. which always took the form of a peak and never that of a plateau. Thereafter it declined to below 1 mg. A typical reducing sugar curve for disks pretreated at 12° C. is shown in Fig. 5. The rapidity with which the peak is attained is determined by the temperature of the pretreatment and examples of the close correlation between reducing sugar content and respiration in disks pretreated at 7° C., 17° C., and 25° C. are shown in Figs. 6–8.

Paper chromatograms confirmed the quantitative findings and established the reducing sugars as glucose and fructose. Initially only a trace of each reducing sugar was present but gradually the glucose spot became more and more pronounced as the concentration of reducing sugar built up. The intensity of the fructose spot remained practically unchanged. The sucrose spot which at first was very well developed testifying to an abundance of sucrose gradually diminished until glucose alone remained. When the disks were 4 days old there appeared on the chromatogram a spot which from its position was probably a trisaccharide. Although this spot persisted until the disappearance of the sucrose it did not increase in concentration. The spot consistently ran a significant fraction farther than raffinose and its position relative to raffinose and sucrose suggested the possibility that it might be kestose (Bacon and Edelman, 1950).

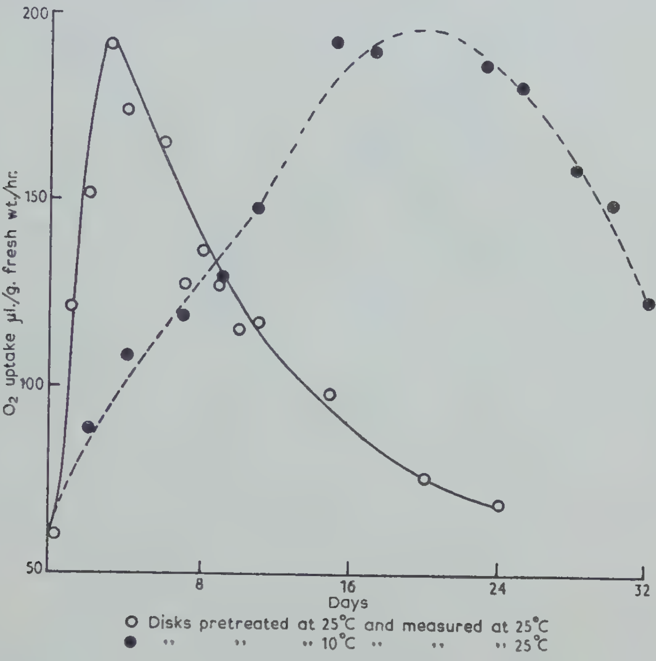

Fig. 4. Respiratory drift of sugar-beet disks.

Red beet: The carbohydrate and respiratory drifts of disks from red beet followed a pattern identical with that of sugar-beet. Fig. 9 illustrates the pattern obtained from disks pretreated and measured at 15° C. and Fig. 10 again shows the higher peak and telescoping effect obtained from disks pretreated and measured at 25° C. As in sugar-beet there is a very close correlation between reducing sugar content and respiratory rate. Total sugar, the initial concentration of which was 55 mg./g./fresh wt., fell continuously and more rapidly in the disks pretreated at 25° C.

Carrot: The carbohydrate and respiratory patterns for carrot disks pretreated and measured at 12° C. and 25° C. are shown in Figs. 11 and 12. Carrot differs from beet in that in the freshly cut disks about 25 per cent. of the total sugar present (50 mg./g. fresh wt.) is in the form of reducing sugar. However, it appears from the quantitative estimations that there is some conversion of sucrose to glucose and fructose and there is substantially the same parallelism between respiration and reducing sugar as appeared in beet. In the disks maintained at 25° C. the respiration attained maximum intensity within 24 hours.

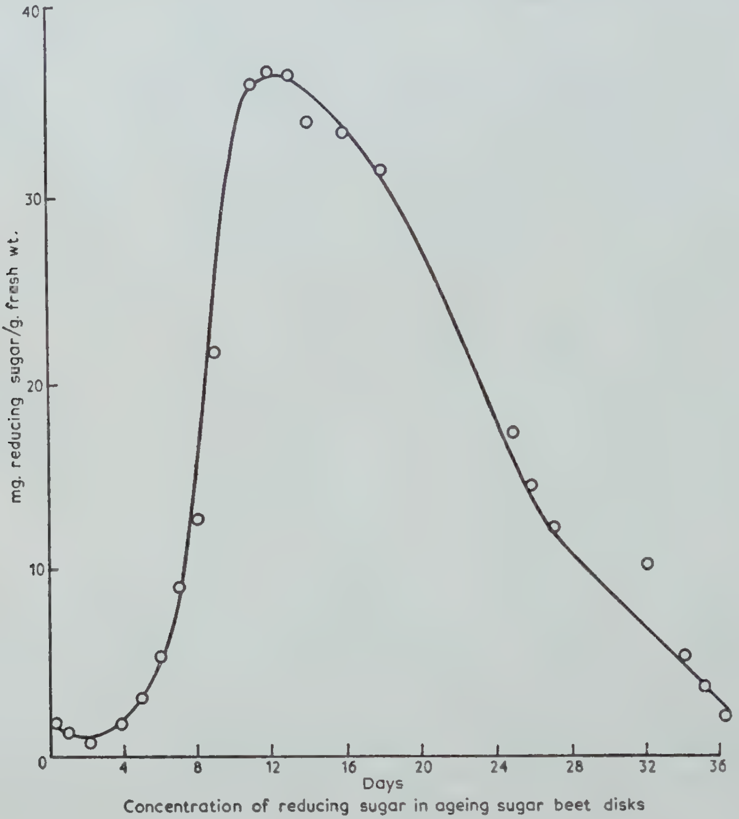

FIG. 5. Concentration of reducing sugar in ageing beet disks.

Swede: This tissue differs from those previously mentioned in that its total sugar (approximately 50 mg./g. fresh wt.) consists almost entirely of reducing sugar, glucose being predominant. Consequently reducing sugar follows that steady fall pattern during ageing obtained for total sugar from beet and carrot. This pattern and the respiratory drifts for disks pretreated at 7° C. and 25° C. and measured at 25° C. are shown in Figs. 13 and 14. The close parallelism observed with the previous tissues is not shown by swede. Disks pretreated at 25° C. reach a maximum value in 24 hours but this is not accompanied by a corresponding increase in reducing sugar. The divergence between respiration and sugar is more apparent with disks pretreated at 7° C. where a steadily increasing respiration rate is accompanied by a decreasing sugar concentration.

Potato: Respiratory and carbohydrate patterns as shown in Figs. 15 and 16

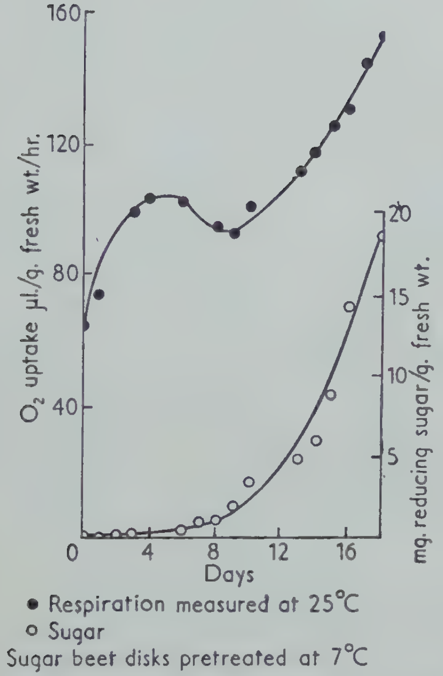

FIG. 6

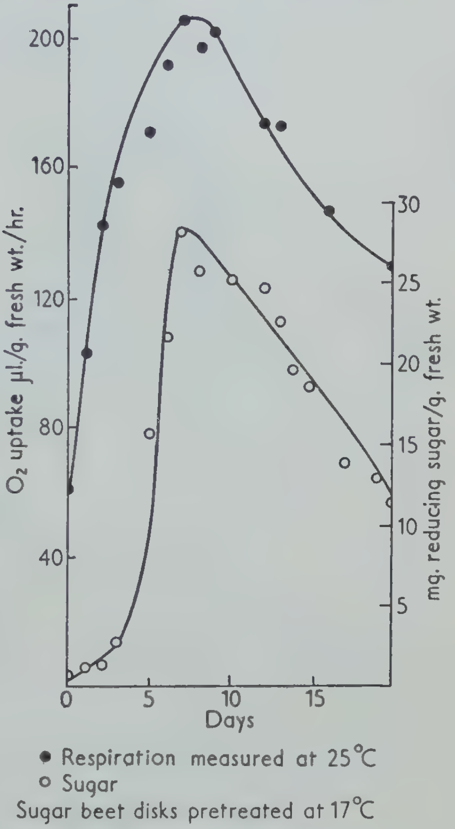

FIG. 7

FIG. 6. Respiration and reducing sugar values in sugar-beet disks pretreated at 7° C.
FIG. 7. Respiration and reducing sugar values in sugar-beet disks pretreated at 17° C.

were obtained from potato disks pretreated at 12° C. and 25° C. and measured at 25° C. Disks maintained at the higher temperature attained a maximum respiratory intensity after 4 days. Both total and reducing sugar increased in concentration doubtless due to the hydrolysis of starch, the increase in the cold disks being greater and more prolonged although less rapid. Paper chromatograms showed that some sucrose was present in the early stages of incubation but glucose was the sugar present in greatest concentration.

RESPIRATORY RATES AND DRIFTS

Since the turn of the century tissue slices have been extensively used in physiological research but the majority of the earlier investigators were concerned exclusively with problems of salt uptake. Respiration studies, if pursued at all, were incidental and results consequently fragmentary. Although the classical researches by F. F. Blackman and his students stimulated widespread interest in the respiratory drifts of leaves and fruits serious investigation of the respiratory activity of disks used in absorption studies was deferred until F. C. Steward directed attention to the causal relationship between respiration and salt accumulation. Since then the respiratory drifts of disks from different storage organs have been investigated by different workers and a review of the now extensive literature leaves the impression that as regards respiratory patterns there is neither uniformity between tissues nor unanimity between investigators.

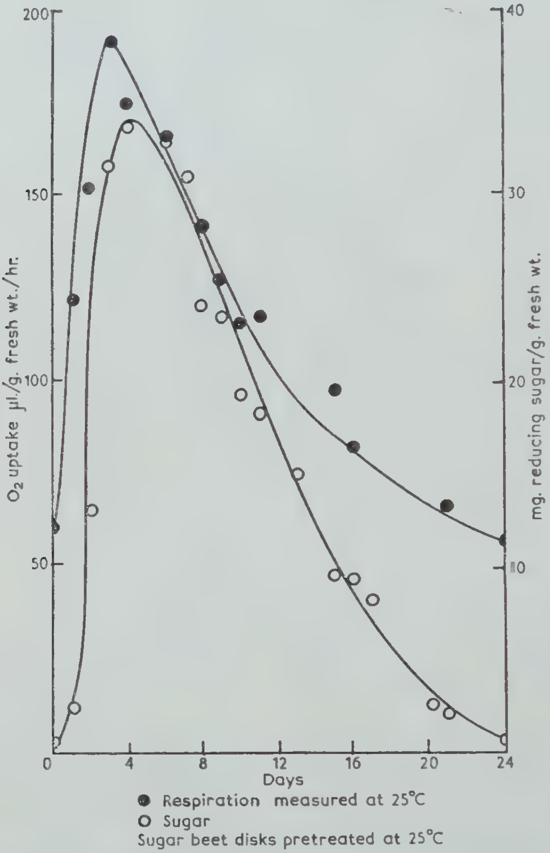

FIG. 8. Respiration and reducing sugar values in sugar-beet disks pretreated at 25° C.

Berry and Steward (1934) made continuous measurements over a period of 100 hours at 23·2° C. of the respiration rate of disks of carrot, parsnip, beet, kohl-rabi, turnip, artichoke, dahlia, and potato. Of these tissues artichoke and potato exhibited a progressively declining respiration rate and kohl-rabi a steadily increasing rate, while the others occupied intermediate positions between these two extremes. Since then more intensive and prolonged investigations of the respiratory drifts of some of these tissues have been carried out.

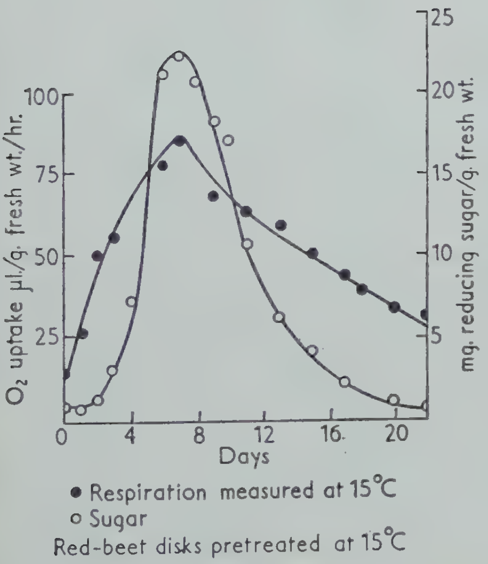

FIG. 9

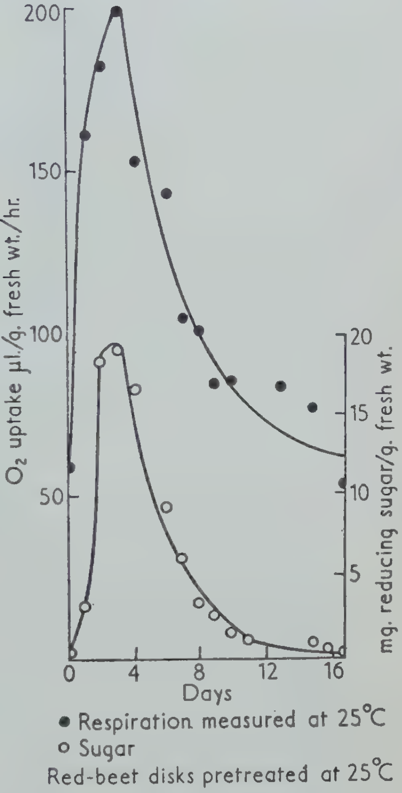

FIG. 10

FIG. 9. Respiration and reducing sugar values in red-beet disks pretreated at 15° C.
FIG. 10. Respiration and reducing sugar values in red-beet disks pretreated at 25° C.

Carrot: Turner (1940), on the basis of Pettenkofer experiments, concluded that carrot disks exhibit their highest respiratory activity immediately after cutting. Their respiratory time-drift follows the general pattern of an exponentially decreasing curve. Robertson, Turner, and Wilkins (1947) present a typical time-drift which depicts a maximum respiratory rate at about 50 hours, after which a gradual decline sets in. Turner and Hanley (1949) obtained basically similar time-drifts. Stiles and Dent (1947) obtained completely different results from their carrot disks in which respiration steadily increased for 214 hours. They suggested that the discrepancy between their results and Turner's might be accounted for by differences in the composition of the tap water, the variety of carrot, its age, or conditions of storage. Our results, however, incriminate temperature of pretreatment as the primary cause. Turner's disks were kept in a continuous current apparatus in which the temperature was maintained at 22·5° C., whereas Stiles and Dent pretreated their disks in running tap water at 12° C. By reproducing these temperature conditions we have shown that disks maintained at 25° C. reach a respiratory maximum in 24 hours, whereas the respiration of disks pretreated at 12° C. continues to rise for almost a week.

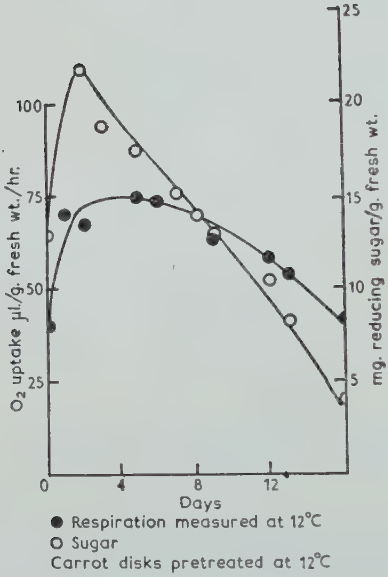

FIG. 11

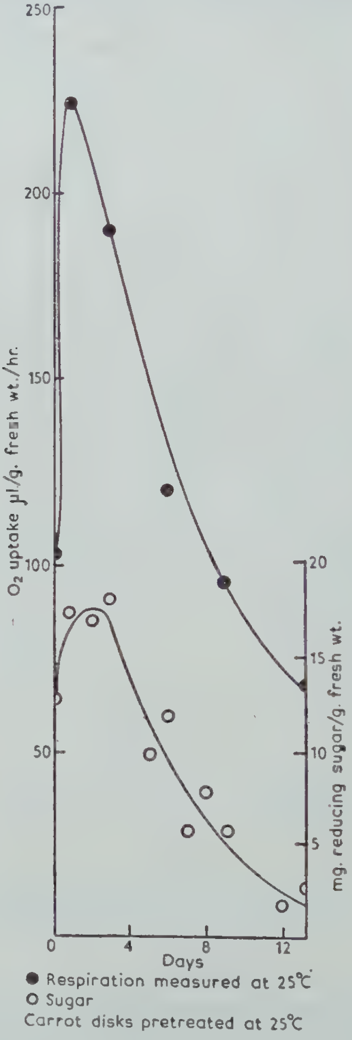

FIG. 12

FIG. 11. Respiration and reducing sugar values in carrot disks pretreated at 12° C.
FIG. 12. Respiration and reducing sugar values in carrot disks pretreated at 25° C.

Red beet: Bennet-Clark and Bexon (1943) followed the respiratory drift of red-beet disks manometrically and reported that the respiration rate rose rapidly on ageing to reach a maximum value at about 300 hours, after which it remained constant for a further 300 hours. Their results are in substantial agreement with those of Stiles and Dent (1947) who obtained a maximum value at about 9 days. This peak was followed by a gradual decline. On the other hand, Robertson, Turner, and Wilkins (1947) obtained a maximum respiratory value within 30 hours of cutting, a result which they found 'difficult to reconcile' with that of Bennet-Clark and Bexon. As with carrot tissue it would seem that the apparent discrepancy between these results can be explained by differences in the temperature of pretreatment. Bennet-Clark and Bexon pretreated their disks in aerated running tap water at a temperature

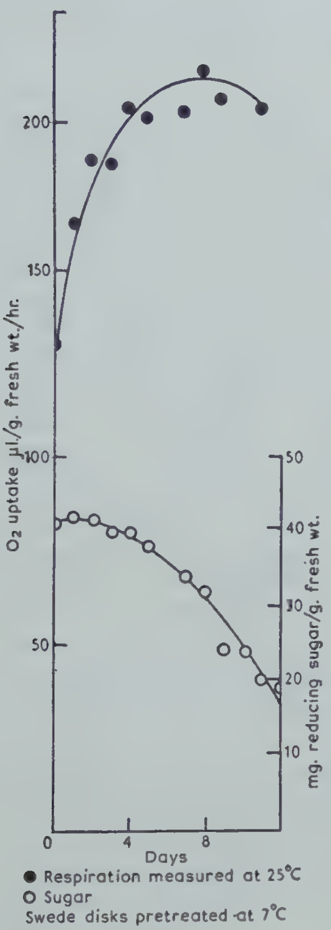

FIG. 13

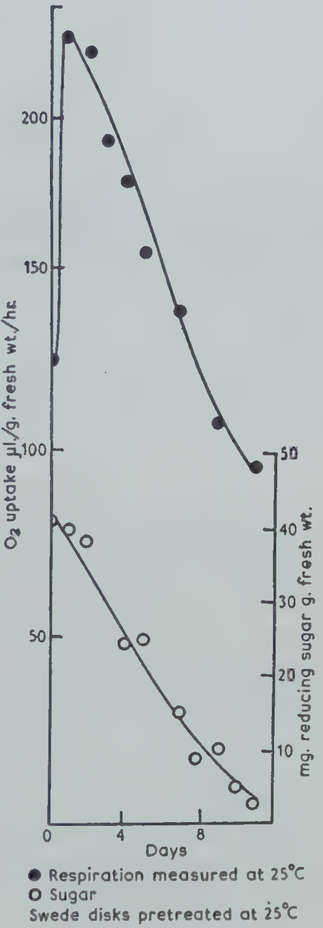

FIG. 14

FIG. 13. Respiration and reducing sugar values in swede disks pretreated at 7° C.
FIG. 14. Respiration and reducing sugar values in swede disks pretreated at 25° C.

between 12° C. and 16° C. and Stiles and Dent maintained their disks in running water at 12° C. Robertson *et al.*, however, kept their disks in aerated distilled water. No indication is given as to how often the water was changed and presumably washing was not continuous and the disks were therefore pretreated at room temperature. We have confirmed that disks pretreated at 25° C. reach a maximum value in about 50 hours and thereafter a very rapid fall sets in, whereas disks pretreated at 12° C. require at least a week to attain their maximum value and the subsequent decline is more gradual.

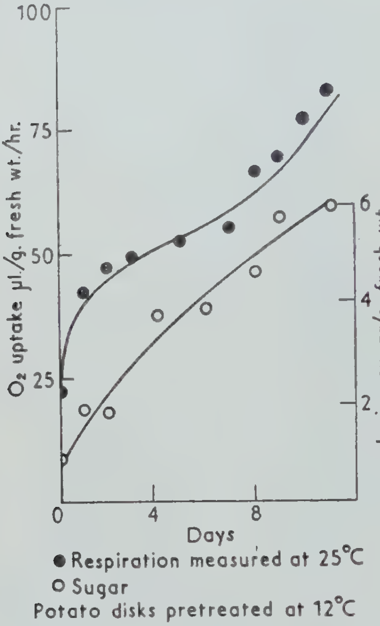

FIG. 15

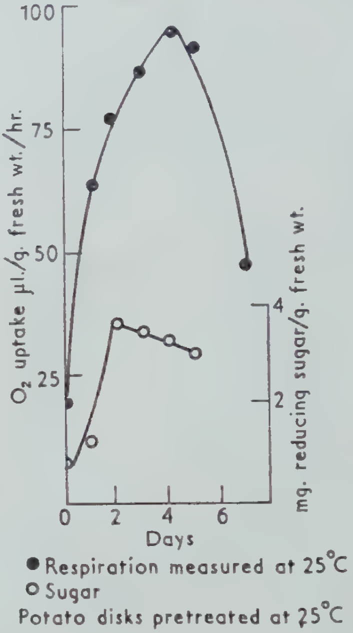

FIG. 16

FIG. 15. Respiration and reducing sugar values in potato disks pretreated at 12° C.
FIG. 16. Respiration and reducing sugar values in potato disks pretreated at 25° C.

Potato: Steward, Wright, and Berry (1932) followed the respiration of disks at 23·2° C. over a period of 140 hours in which time they recorded 2 respiratory maxima. Boswell and Whiting (1938) obtained similar results. Schade and Levy (1949) followed the respiration rate over 100 hours of disks which had been maintained in running tap water at 14° C. and their graphs depict progressively increasing respiratory activity. Mulder (1955) measured manometrically the respiratory intensity of potato disks respiring in moist air at 25° C. He found that the rate of O_2 uptake increased very rapidly for 55 hours, whereupon it fell away just as rapidly over the next 60 hours. Our results suggest that the findings of Mulder and Schade and Levy are generally typical of the respiratory pattern of potato disks. The respiratory rate of disks maintained at 25° C. rapidly intensifies to achieve a maximum value in 4 days, and thereafter it declines with an equal rapidity. The respiratory increase in disks kept at 12° C. is slower and may be protracted over a period of 10 days, during which the increase seems to develop in two distinct phases.

Swede: Boswell (1950) carried out an investigation on metabolic systems in swede disks but did not record any information on respiratory time-drifts. Berry and Steward (1934) showed that the CO_2 output of disks maintained at 23·2° C. and estimated over 100 hours increased steadily for 72 hours and then decreased. Our results with swede disks are identical with those of carrot disks.

Other tissues: Berry and Steward (1934) and Steward, Berry, and Broyer (1936) have shown that the time-drift of artichoke disks follows a continuous decline. Said and El Shishiny (1947) measured the CO_2 output of radish disks at 25° C. by a continuous current method and showed that immediately after cutting the respiration rate rapidly increased, attaining a maximum level after 20 hours. The subsequent fall was equally rapid and after 60 hours had reached a level only slightly higher than that of the uncut root. Stiles and Dent (1947) state that the respiratory pattern for mangold disks pretreated at 12° C. and 16° C. is similar to that of red beet disks although their published results show only an increase extending over 14 days. Berry and Steward (1934), however, show a slight fall after an initial rise in the CO_2 output of mangold disks kept at 23·2° C.

These results exhaust the published reports of respiratory time-drifts in disks of storage tissue that have come to our notice. No previous work appears to have been done with sugar-beet tissue the respiration of which we have found to be identical with that of red-beet disks.

Irrespective of the temperature of pretreatment disks cut from the five tissues under consideration all show an initial respiratory increase. The sequential respiratory pattern is, in its broad outline, determined by the temperature of the pretreatment and is independent of the temperature of measurement, although the latter temperature of course determines the absolute values recorded (Fig. 3). Disks of carrot and swede pretreated at 25° C. attain their maximum value within 24 hours, but disks of sugar-beet, red beet, and potato all take more than one day to reach this peak value. The earliest peak recorded was 3 days after pretreatment at 25° C. and with pretreatment at lower temperatures down to 7° C. the peak could be delayed for 3 weeks.

DETERMINANTS OF THE RESPIRATORY TIME-DRIFT

The outstanding feature of disk metabolism is the immediate rise in respiration following dissection from the storage organ. Excision appears to occasion a radical alteration to the normal metabolism of the tissue, an alteration having profound and far-reaching consequences transforming quiescent relatively inactive tissue into a flux of dynamic metabolic activity which sooner or later results in its own complete exhaustion. What precisely is the nature of the stimulus leading to the acceleration of vital activities manifested most prominently in the altered respiration remains a matter for speculation and experiment, but a number of suggestions have been put forward.

Handling: A period of increased respiration is apparently a feature of widespread occurrence in excised tissues and there is some evidence to show that a respiratory stimulus results from manipulation of leaves (Godwin, 1935; Audus, 1939; Arney, 1947) and potatoes (Mulder, 1955). Turner (1940) has suggested that the increased respiration rate of carrot disks is partially attributable to manipulation and Steward (1932) showed that potato disks were sensitive to mechanical agitation. On the other hand, Stiles and Dent (1947) suggest that contact stimulation is of very secondary importance and Bennet-Clark and Bexon (1943), in the course of their experiments with red-beet tissues, actually removed the disks from the Warburg vessels (which presumably involved considerable handling) but showed that on replacement in the vessels the respiration of the disks was equal to that of control disks left undisturbed.

Release of cellular substances: Steward, Wright, and Berry (1932) and Bennet-Clark and Bexon (1943) among others have postulated wound stimuli as inducing an augmented respiration rate. Historically this view has been associated with the concept of a 'wound hormone', although that is not a necessary inference. It may be, however, that cutting does release cellular substances which stimulate metabolic activity in the tissue. If this were so, washing of the disks would tend to reduce the wound respiration, and Steward, Wright, and Berry (1932) have shown that such is the case, although they attributed the decreased rate in potato disks respiring in air after preliminary washing compared with unwashed disks to impaired aeration of the tissue rather than to the removal of stimulatory substances. Stiles and Dent (1947) similarly showed that the shorter the washing period the greater and the more immediate was the respiratory increase. We have noticed that the most rapid respiratory increase in sugar-beet tissue is found in disks washed in unchanged distilled water in which no provision is made for the removal of cell exudates. This suggests that substances released as a consequence of wounding can stimulate respiration, and other workers have shown that the application of tissue sap to disks increases their respiration rate (Ruhland and Ramshorn, 1938; Bennet-Clark and Bexon, 1943). Dale and Sutcliffe (1956) have claimed that the stimulatory effect on respiration of an extract of red-beet tissue (Skelding and Rees, 1952) is largely attributable to the presence in the extract of respirable molecules, mainly sugars and organic acids.

Changes in gaseous environment: A higher oxygen tension has frequently been suggested as the cause of the increased respiratory activity of disks, but a comprehensive discussion of this subject in a recent review (Laties, 1957) obviates the need for a detailed treatment of it here. Laties has assembled a formidable body of evidence to show that the low level of respiratory activity in uncut storage tissue relative to that developed in disks cannot be ascribed to an oxygen deficit in the bulky storage tissue. Burton (1950) is quoted to show that at storage temperatures between 5° C. and 15° C. the tissue fluid of potato is in apparent equilibrium with a gas mixture containing 17 per cent. to 19·5 per cent. of oxygen, and at 25° C. the liquid phase is still in equilibrium with the internal gas phase containing 12 per cent. oxygen, and this despite the fact that the gas phase frequently constitutes 1 per cent. or less of the tissue volume. Since the relationship between oxygen tension and respiratory activity can be depicted as an exponential curve with the rate in air approaching a near maximum and since the partial pressure of oxygen in thin disks is at best only double that in uncut root the difference in oxygen tension cannot account for a tenfold respiratory increase (found in potato disks) or a fivefold increase as in carrot disks or even a twofold increase. In other words the rapid respiratory rise in sliced tissue is not simply a consequence of a higher oxygen tension. The increased oxygen tension may subsequently permit the attainment of a still higher respiratory level in the disks, but it does not induce it. Recently Mulder (1955) has shown that wounding itself and not an improved O_2 supply is responsible for the initial large increase in respiration of sliced tissue by sealing, after a quick rinse, the cut surfaces of potato tissue with paraffin wax which failed to prevent the development of the increased respiration. Scott (quoted in Goddard and Meuse, 1950) showed that the CO_2 content in beet could be as high as 10 per cent. and suggested that the suppression of respiration in uncut root might be caused by a high internal carbon dioxide pressure. Laties (1957) claims to have tentatively eliminated CO_2 as the agent responsible for the respiratory limitation.

Development of an inhibitor: Steward, Wright, and Berry (1932) showed that the specific respiratory activity per unit weight of potato disks decreased with decreasing surface : volume ratios and attributed this decrease to limitations in the rate of oxygen diffusion to the internal cells. Laties (1957), however, observed that the respiration of freshly cut disks was independent of thickness and that the relationship referred to by Steward *et al.* only became apparent in disks which had been aged for 24 hours. Mulder (1955) has also shown that the respiration rate of young potato disks is proportional to the weight of the disks and not to their surface area. Only as the disks aged did a surface effect become apparent, which effect Mulder ascribed to the possibility that sealing of the wounds hampered O_2 uptake. Laties (1957), by slicing disks aged at different thicknesses, has demonstrated that it is the development of the respiratory system which arises on ageing that is conditioned by disk thickness and not the manifestation of that system once it has developed. The experiments of Steward *et al.* and Mulder were obviously carried out on disks which had been aged at different thicknesses and so the observed effects of thickness on respiration rate reflected the relationship between disk thickness and the development of respiratory activity. To account for this latter relationship Laties postulated the existence of a volatile metabolic product the effect of which was inhibitory to the development of the respiratory increment consequent to wounding. This compound would also be responsible for the suppression of the respiratory rate in uncut root. Skelding (1957) suggests that the depression of metabolic mechanisms in bulky storage roots is due to an inhibiting compound formed by the dark fixation of accumulated respiratory carbon dioxide.

Substrate concentration: James (1953) reviewed the extensive literature dealing with respiratory drifts and carbohydrate analyses in leaves, seedlings, and fruits and observed that in none of these tissues was there an intimate relationship between the rate of respiration and the concentration of any one sugar. Boswell (1950) could find no evidence to support the view that sugar content was the limiting factor in the respiration of sliced tissue, and Laties (1957) considered that substrate level was not the primary factor limiting respiration in tissue disks. However, wounding or excision of potato tissue has for long been known to be accompanied by changes in the carbohydrate reserves. Hopkins (1927) found that wounding of potato tissue caused the hydrolysis of starch and he postulated a correlation between cellular sugar concentration and respiration. Steward reported from his studies with potato disks that the surface cells undergo starch hydrolysis (Steward, Wright, and Berry, 1932) but he concluded from total sugar analyses of the disks that total sugar concentration was not the factor controlling the respiration rate and he thought it improbable that the concentration of any individual sugar would show a more positive correlation (Steward, Stout, and Preston, 1940; Steward and Preston, 1941). Steward, Preston, and Ramamurti (1943) showed that the respiration (as reflected in 2-day-old disks) of potatoes stored at 11° C. was more nearly parallel to the concentration of the reducing sugar than to the disaccharide fraction. Stiles and Dent (1947) suggested that the respiratory increase in disks might be due in part to a change in metabolic activity caused by a shift in the carbohydrate equilibrium of the cell resulting in an increased concentration of actual substrate.

In sugar-beet disks, respiration bears no direct relation to total sugar since it is at its lowest in the freshly cut disks when sucrose concentration is highest and for a time steadily rises while the concentration of total sugar is falling. However, there appears to be a positive correlation between respiration and concentration of reducing sugars since they rise and fall together (Figs. 6–8). During sucrose consumption there is a temporary increase in hexose. Chromatographic analyses have established the fact that glucose rapidly accumulates while there is little change in the concentration of fructose. Unless therefore there is a rapid interconversion of fructose to glucose the fructose is evidently rapidly respired and it is significant that the maximum rate of respiration coincides with the maximum concentration of hexose. Feeding with sugars has already been shown to increase the respiration of tissue disks (Turner, 1938, 1940; Said and El Shishiny, 1944; Sutcliffe, 1954). However, some preliminary experiments (MacDonald, 1955) with 2:4-dinitrophenol, which stimulates O_2 uptake even at its maximum intensity, show that respiration is not entirely regulated by the concentration of effective substrate, but these experiments also show that the concentration of reducing sugar does bear some relation to the rate of respiration since the rate in the presence of DNP increases with time as the concentration of reducing sugar increases.

Steward has previously reported the increase in reducing sugar in potato disks, but we are not aware of any earlier reports of sugar changes in carrot, red-beet, or sugar-beet disks. These four tissues all show a positive correlation between oxygen uptake and reducing sugar concentration. This correlation does not appear in swede disks.

CONCLUSIONS

Although the five tissues used in this investigation differ anatomically and possibly physiologically they show a similarity in their immediate reaction to excision which suggests that the response is conditioned more by the character of the stimulus than by the nature of the tissue. This response is not peculiar to plant tissue. Wounding of animal tissue is also accompanied by intensified respiration. However, the pattern of events occurring in the disks cannot be ascribed simply to a traumatic stimulus because of the transient nature of such a stimulus. What could properly be termed wound respiration might well contribute to the respiratory increase in its early stages but the prolonged development of respiratory activity, which Laties (1957) terms the 'induced respiration' as distinct from the 'basal respiration' of the uncut tissue, must be occasioned by internal changes in the cell which can be regulated to some extent by external conditions. It may be that the early development of the induced respiration is possible without gross changes in the disk, but its complete development is limited by internal factors one of which is the concentration of effective substrate.

Fig. 6 relating to sugar-beet disks maintained at 7° C. shows that during the first week, when there was no increase in reducing sugar content, the respiration increased by 50 per cent., after which it fell away slightly, and only when a build-up of reducing sugar occurred did the respiration attain the usual intensity. During the first week the loss in total sugar was negligible, so that the absence of any increase in hexose sugar was not due to its continued utilization in respiration. In other words the early development of the induced respiration occurs independently of gross sugar changes, but its complete development is conditioned by internal changes of which one is an increase in the effective substrate. Figs. 5, 7, and 9 also show that substantial respiratory increases can occur in the early stages apart from large-scale sugar changes. The internal changes that determine the general pattern of the disk respiration are obviously temperature-controlled, so that the exact form of the respiratory time-drift is determined by the temperature of pretreatment.

It may be significant that disks of sugar-beet, red beet, and potato, in which the reducing sugar content commences at a level below 1 mg./g. fresh weight, require about 3 or 4 days to reach their respiratory maximum even when maintained at 25° C., whereas similarly treated disks of swede and carrot, which contain a substantial concentration of glucose when cut, reach their maximum intensity within 24 hours. There is no reason to assume that the rapid increase in carrot and swede is purely wound respiration and that no induced respiration occurs because if the disks are pretreated at a low temperature both tissues, and particularly swede, manifest a more gradual respiratory development. The fact that swede disks show a respiratory development without any increase in hexose sugar is not surprising because obviously since hexose sugar is the storage sugar there must be some mechanism preventing it from being uncontrollably respired. It is of course true of all tissues that sugar is generally segregated from the metabolic pool and the feature common to the sugar changes in the tissues under consideration in this paper is not the transformation of sugars but their being rendered increasingly available to the respiratory centres. If the increase of sugar availability is not the underlying cause of the increased respiration, it at any rate permits the development of that respiration and determines the general pattern of the time-drift.

The surface: volume effect apparent in the respiratory activity of disks of different thicknesses has been interpreted by Laties (1957) as indicating the presence of a volatile inhibitor. Another possibility is that the bulk of the respiratory activity is effected by the outer cells and that the induced respiration is limited to these cells because the sugar changes are confined to the outer cells. Said and El Shishiny (1944) claim that the rate of carbohydrate disappearance increases with decreasing thickness of radish slices, and Hopkins (1927) and Steward, Wright, and Berry (1932) showed that starch depletion and hydrolysis was limited to the surface cells of potato disks. Such differential sugar changes in disks of varying thicknesses could account for the surface: volume effect.

The metabolic changes induced by excision in disks of storage tissue and manifested most prominently in the altered respiration all, in the last analysis, resolve themselves into the question of rate control. The normal regulatory mechanism of storage tissue undergoes some profound disturbance which in effect precipitates everything into a maelstrom of metabolic activity. By maintaining the disks in suitable conditions, i.e. in running aerated water at a temperature around 12° C. rather than in moist air at a high temperature, it is possible that the chemical energy unleashed by the intensified metabolism may be harnessed to synthetic processes resulting in the continued production of phosphate acceptors which are known to exert a regulatory influence on respiratory processes. Such a recrudescence of synthetic activity would permit the progressive development of respiration manifested by disks in favourable conditions. It will be the purpose of a subsequent paper to show that the controlled respiratory development is in fact accompanied by synthetic processes.

ACKNOWLEDGEMENTS

The experiments with sugar-beet disks were carried out during the tenure of an A.R.C. maintenance grant to I. R. M. who gladly acknowledges his indebtedness to the Agricultural Research Council and to Dr. D. N. McArthur, Director of the Macaulay Institute for Soil Research, for research facilities.

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Extension growth in Primary Cell-Walls with Special Reference to *Hippuris vulgaris*

BY

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With one Plate and four Figures in the Text

Abstract

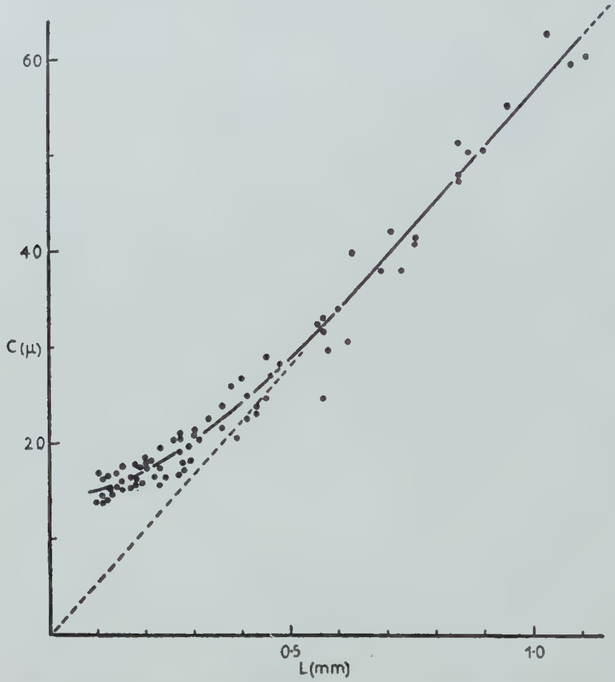
A quantitative study of pit-field distribution in the longitudinal walls of cortical parenchyma shows that pit-fields increase in number continuously during cell division and growth. A consideration of the data in relation to other information on pit-field distribution and to wider aspects of cell-wall extension suggests that pit-field multiplication may be expected to be a characteristic feature of the growth of intercellular cell-wall surfaces in meristem cells.

In a previous communication (Wilson, 1957) attention was drawn to the distribution of pit-fields in the longitudinal cell-walls of the cortical parenchyma of *Elodea* shoots. It was shown, from measurements on internodes of lengths ranging from about 0·25 mm. to 12 mm., that the spacing of the pit-fields increases during growth in an orderly manner, which precludes the application of the hypothesis of tip-growth to the cells of this tissue; cell growth must be visualized as taking place over the whole area of the wall. The view that the pit-field perforations have a specific function in relation to the extension growth of the cell-wall, as proposed by Frey-Wyssling and Stecher (1951) and Stecher (1952) in their hypothesis of 'mosaic growth' was also held to be invalid. These investigators considered pit-fields as sites of transient breakdown and new synthesis of the cellulose framework of the wall. In *Elodea*, however, the perforations are present in very young internodes before extension growth has got under way, and the greater part of the cell-wall extension takes place between them. Furthermore the cell-walls abutting on to intercellular spaces lack them altogether.

There is, however, another aspect of this changing pit-field distribution which merits further attention. In the previous work pit-field spacing was considered quantitatively in relation to internode length (i.e. to the elongation of the tissue as a whole) rather than in relation to the elongation of individual cells, since in this way certain aspects of the changes which occur could be more simply displayed, uncomplicated by considerations of cell division. It was shown that, in the early stages of the growth of an area of cell-wall the number of pit-fields it bears increases. This increase in pit-field numbers results apparently from the division of existing pit-fields, brought about by the intercalary growth of thin strands traversing them. During the final stages of cell-wall elongation, however, the number of pit-fields tends to remain

[Annals of Botany, N.S. Vol. 22, No. 88, Oct. 1958.]

constant. This was interpreted as indicating that cell-wall growth now takes place between existing pit-fields without new ones being formed. The purpose of the present note is to consider this change in the behaviour of the growing wall further, in the light of similar data derived from the corresponding tissue in *Hippuris vulgaris*. *Hippuris* shares with *Elodea* those features of structure of which use was made in the previous work. The internodes are short, and near the stem-apex form a series closely graded in length, so presenting a convenient measure of the degree of elongation of the tissue, since its initiation, at successive stages of its development. The cortical cells are prismatic in shape and arranged in regular vertical rows so that intercellular relationships are relatively simple. There is, however, a difference in the modes of growth of the cortices of the two stems which is important in the present context, as will be shown.



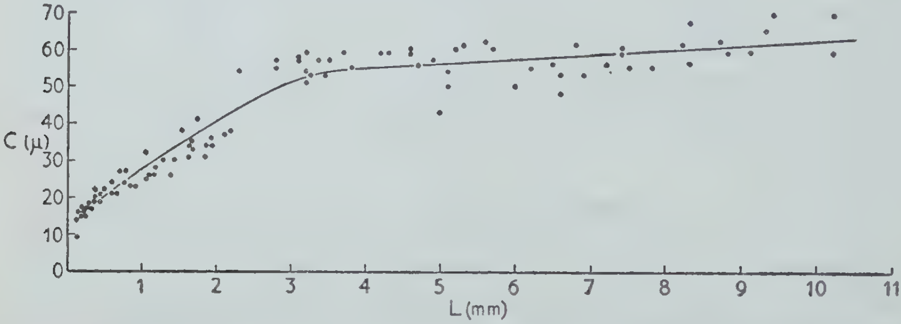
TEXT-FIG. 1. *Elodea canadensis*. The relationship, in cortical parenchyma, between internode length (L) and mean cell length (C). For internodes longer than about 0·6 mm. C is directly proportional to L, indicating a cessation of cell-division.

It is necessary first, however, to revert to the earlier observations on *Elodea*, and to relate the changing pit-field distribution to cell elongation and to the incidence of cell division, as well as to the elongation of the tissue as a whole. Cell division is generally distributed throughout the internodal cortex, and is not limited to sharply defined intercalary meristems. The extent to which it takes place in internodes of increasing length may, therefore, be indicated in graphical form in the manner of Text-fig. 1. Here is displayed, over the interesting range of internode lengths, cell length (mean of 50–100 cells in each internode considered) plotted against the length of the internode in which they occur. It is clear from the graph that, in internodes of lengths up to about 0·6 mm., mean cell length increases less rapidly than internode length; i.e. extension growth is a resultant, not only of the elongation of cells already formed, but also of the formation of new cells. For internodes longer than about 0·6 mm., however, mean cell length is proportional to internode length: a straight line drawn through the points in this region of the graph may be extrapolated back to the origin. In these internodes, therefore, cell division has ceased, and the extension growth of the tissue as a whole may be referred simply to the elongation of existing cells. In mature internodes of lengths about 10 mm. the mean length of the cortical cells is in the region of 600μ.

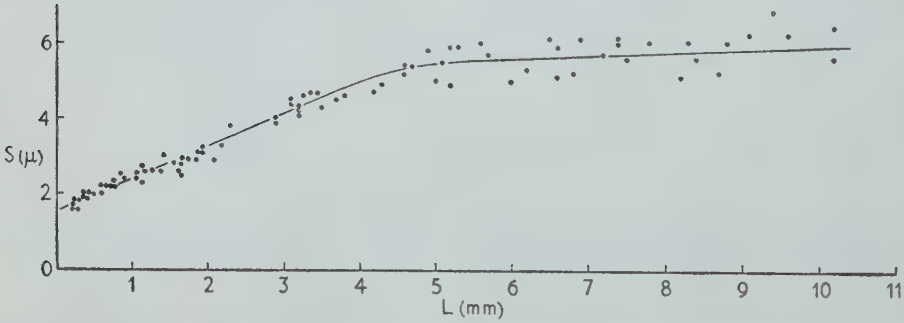
In relation to this it may be recalled that in the same tissue pit-fields increase in number very steeply in relation to internode length in internodes of lengths up to about 1 mm. In longer internodes the increase is less marked, and in internodes longer than about 4 mm. the number of pit-fields remains approximately constant. Thus the phase of most marked increase in pit-field numbers corresponds approximately to the phase of cell-division.

In *Hippuris*, as is well known, the stem structure generally is on a much larger scale than that of *Elodea*: the cortex is larger and more lacunar, but exhibits a general similarity in make-up to that of the smaller plant. The cortical parenchyma consists almost entirely of vertical plates of closely fitting prismatic cells, one cell thick, which form the walls of the numerous schizogenous air-filled intercellular spaces. These are polygonal in section and extend from node to node. As in *Elodea*, the cell-walls of the cortical parenchyma which abut on to the air-spaces show no microscopic discontinuity of structure: it is with the intercellular walls that we are at present concerned. Unlike those of *Elodea*, however, the cortical parenchyma cells of *Hippuris* do not become greatly elongated in the growth of the stem: the relationship between cell length (mean of 50–100 cells in each internode considered) and internode length is shown in Text-fig. 2, which may thus be compared with Text-fig. 1 for *Elodea*. Though in *Hippuris* there is some increase in mean cell length as the internodes elongate, this can account only for a small fraction of the elongation of the tissue as a whole; cell division goes on throughout the range of internode lengths studied. Thus, as an internode increases in length from 0·2 mm. to 10 mm., i.e. by a factor of fifty, the mean length of its cortical cells increases from about 16μ to 65μ, i.e. by a factor of about four only.

The corresponding data for pit-field spacing in relation to internode length in *Hippuris* are summarized in Text-fig. 3. They were obtained essentially by the same method as that used previously for *Elodea*, i.e. by sampling the pit-field distribution along line transects centred on the faces of the cell-walls, as seen in longitudinal sections. This method is somewhat less satisfactory in its application to *Hippuris* than to *Elodea*, since in *Hippuris* the intercellular cell-wall faces grow transversely as well as longitudinally, so that the pit-fields become more dispersed laterally, and might thus be expected to be less frequently intercepted by an imaginary vertical line on the cell-wall. However, there is an effective compensatory change too, in that the pit-fields (or more precisely, perhaps, the disorientated and thus isotropic regions of cell wall associated with them, which is what one really sees in a polarizing microscope) also elongate laterally. It is clear that the differences to be described in the pit-field distributions in the two plants are real differences and do not arise simply from the method of sampling employed.



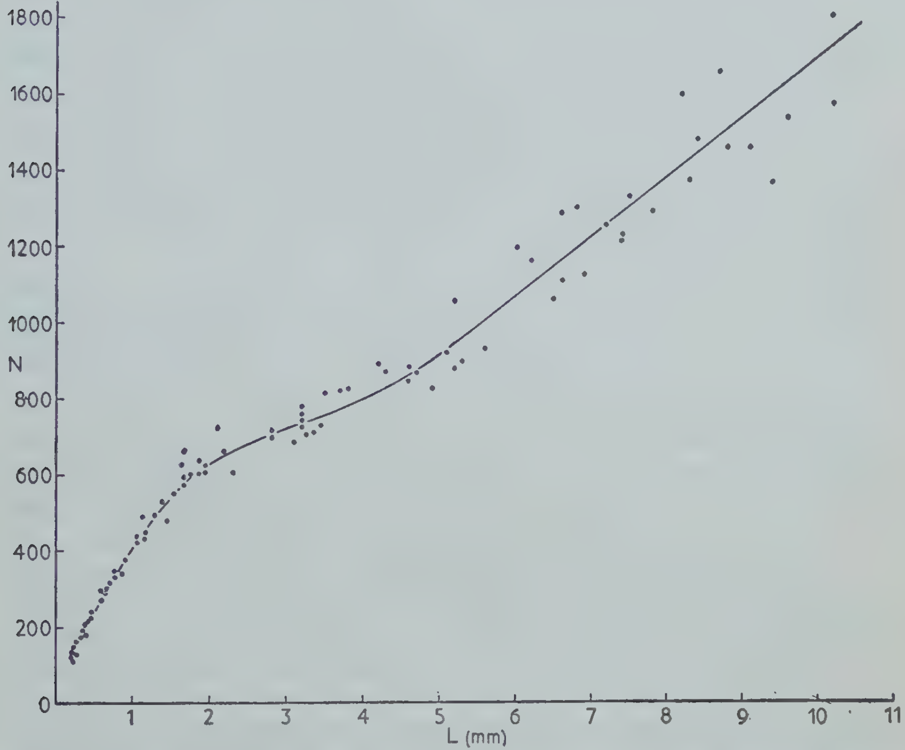
TEXT-FIG. 2. *Hippuris vulgaris.* The relationship, in cortical parenchyma, between internode length (L) and mean cell length (C). The increase of cell length with increasing internode length is relatively small; cell division goes on during the period of growth.



TEXT-FIG. 3. *Hippuris vulgaris.* The relationship, in cortical parenchyma, between internode length (L) and mean pit-field spacing (S) measured along vertical cell-wall transects.

It is evident from Text-fig. 3, which shows mean pit-field spacing plotted against internode length, that the trend of changing pit-field distribution with growth in *Hippuris* differs markedly from that in *Elodea* previously described. Though pit-field spacing increases with increasing internode length, the trend-line of the points is much more nearly parallel to the *L* axis of the graph. The spacing in very short internodes is much the same as in corresponding internodes in *Elodea*, about $1 \cdot 7\mu$, but rises only to about $6 \cdot 5\mu$ in 10-mm. internodes. The corresponding figure in *Elodea* would be about 33μ.

From the data of Text-fig. 3 it is a simple matter to calculate how many pit-fields one might expect, on the average, to find intersected by cell-wall transects extending throughout internodes of various lengths. The results of such calculations are shown in Text-fig. 4, which is thus comparable with Text-fig. 2 of Wilson (loc. cit.) for *Elodea*. It is evident that in *Hippuris*, unlike *Elodea*, the pit-fields increase in number throughout the range of internodes studied (up to 10 mm., approximately), and do not tend to constancy in number within this range. Although the longer internodes commonly to be found near the bases of *Hippuris* shoots (lengths of up to 20–25 mm.) were not studied in detail, inspection indicates that the trends evident in Text-figs. 2, 3, and 4 continue here also. In illustration of these trends, cell-walls from representative internodes, as seen by polarizing microscope in face view in longitudinal sections, are shown in the Plate. These may be compared with those of *Elodea* in Plate 1 of Wilson (loc. cit.)



TEXT-FIG. 4. *Hippuris vulgaris*. The relationship, in cortical parenchyma, between internode length (L) and the mean number (N) of pit-fields along a cell-wall transect extending throughout an internode, calculated from the data of Text-fig. 3.

It is clear from the text-figures and the photo-micrographs that the modes of growth of the cellulose frameworks of the longitudinal walls between cells in the cortices of the two plants follow rather different courses. In the young internodes of both plants the cellulose of the intercellular cell-walls takes the form of a delicate but microscopically visible lattice with transversely elongated meshes; in *Hippuris* this lattice structure is maintained, though in a coarser form, throughout cell-wall growth, but in *Elodea* the lattice-like appearance characteristic of the cell-walls of young internodes is lost in later growth as the result of the intercalation of 'solid' areas of wall between the pit-fields. Associated with this difference in the modes of growth of the cell-walls is a difference also in the modes of growth of the tissue as a whole. In *Hippuris* the cells remain relatively short and meristematic: in *Elodea* cell division ceases at an early stage and subsequent growth is a matter of extension of the cells already formed. Thus there is, in *Elodea* cortex, more or less associated with the cessation of cell division, a change in the mode of growth of the cell-wall, and in the growth both of cell-walls and of the tissue as a whole *Hippuris* cortex persists in a pattern characteristic of *Elodea* only in its juvenile state.

It may perhaps seem unreasonable to emphasize this comparison, since it is one made between plants of no close affinity and whose structural resemblance doubtless stems wholly from a community of habitat. Nevertheless it is worthy of some further consideration in terms of the broad relationship between meristem cells and their mature products. A lattice-like structure of the cellulose component of the cell-wall similar to that described above is probably very general in meristem cells: it has been shown, for instance, in various meristem cells by Muhlethaler (1953), Scott and Lewis (1953), and Scott *et al.* (1956), and inspection, by polarizing microscope, of sections of apices of the following plants which happened to be readily available, shows it to be a feature of the meristems of these too: *Pinus*, *Acer*, *Vicia* (root), *Pisum* (root), *Epilobium*, *Valeriana*, *Helianthus*, *Syringa*, *Glyceria*, and *Agropyron*. Continued division of such meristem cells, if it were not accompanied by a mode of growth of the cell surface which led to increased numbers of meshes in the lattice (i.e. fundamentally, of pit-fields and of plasmadesmata occupying them) would result in the ultimate products of the meristem being virtually devoid of these structures. On the other hand, in cells which have ceased dividing, in which extension growth only is taking place, and furthermore perhaps, in which some slowing of general metabolic activity may be expected, a corresponding increase in pit-field numbers may no longer be of any physiological importance. Hence, perhaps, we find that, not only in the older internodes of *Elodea*, but also in the elongating parenchyma of *Avena* coleoptiles (Wardrop, 1955) in which cell division may be confidently assumed to have ceased (Avery *et al.* 1945) a constancy in pit-field numbers obtains. In *Elodea*, it is true, pit-fields apparently go on increasing in numbers for a while in internodes in which cell division has ceased, but this may well be a consequence of the observational difficulty of detecting the early stages of pit-field divisions. These divisions may become visible only some time after their essential initiation.

Such a point of view, therefore, would imply that during the phase of cell division, growth in area of the cellulose framework of the meristem cell-wall may result not only from the intercalation of new material in existing strands of the microscopically visible lattice structure, but also from the initiation and growth of new strands of material within the pit-fields, so that the pit-fields themselves are divided and increase in numbers. After cell division has ceased, however, this intercalation within the pit-fields also ceases. An hypothesis on these lines may seem to involve a reversion to the concept of 'mosaic growth' in the sense of Frey-Wyssling and Stecher (loc. cit.) and Stecher (loc. cit.), which has been criticized by the present writer (loc. cit.). This, however, is not really the case. The suggestion, now renewed, that the pit-fields take a definite part in cell-wall growth is a radically different one from that proposed by these investigators. It is suggested that the pit-fields are involved in the growth processes of the intercellular cell surface not simply because they are temporary regions of local loosening and resynthesis of the cellulose framework of the cell-wall, by means of which this framework necessarily grows, but rather because they are characteristic morphological features of the intercellular cell-walls and their multiplication as features of the growth of these walls is of the same importance as the growth of the walls themselves. If this is so it would account for the interpretation of the role of pit-fields in cell-wall growth arrived at by Frey-Wyssling and Stecher on the basis of their electronmicroscopic studies, and it resolves, in a sense, the conflict between their evidence and that based upon larger-scale considerations of the cell as a whole and part of a tissue.

SUMMARY

Quantitative estimates of pit-field distributions in longitudinal cell-walls of the cortical parenchyma of *Hippuris*, which show that the pit-fields increase very markedly in number during growth, are compared with similar data, previously reported, for *Elodea*. The differences are considered to be related to differences in the modes of growth of the two tissues. In *Hippuris* cortex growth is dependent upon continued cell division, while in *Elodea* cortex the phase of cell division is a restricted one, and is followed by very marked cell extension. Correspondingly, in *Hippuris* cortex pit-field numbers increase steadily with over-all growth, while in *Elodea* cortex, after increasing in numbers at first they later tend to a constant maximum. These facts are briefly discussed in relation to the general problem of cell-wall growth: pit-fields are considered as characteristic morphological features of intercellular cell-walls and it is suggested that their multiplication in meristem cells is therefore an important feature of cell-wall growth.

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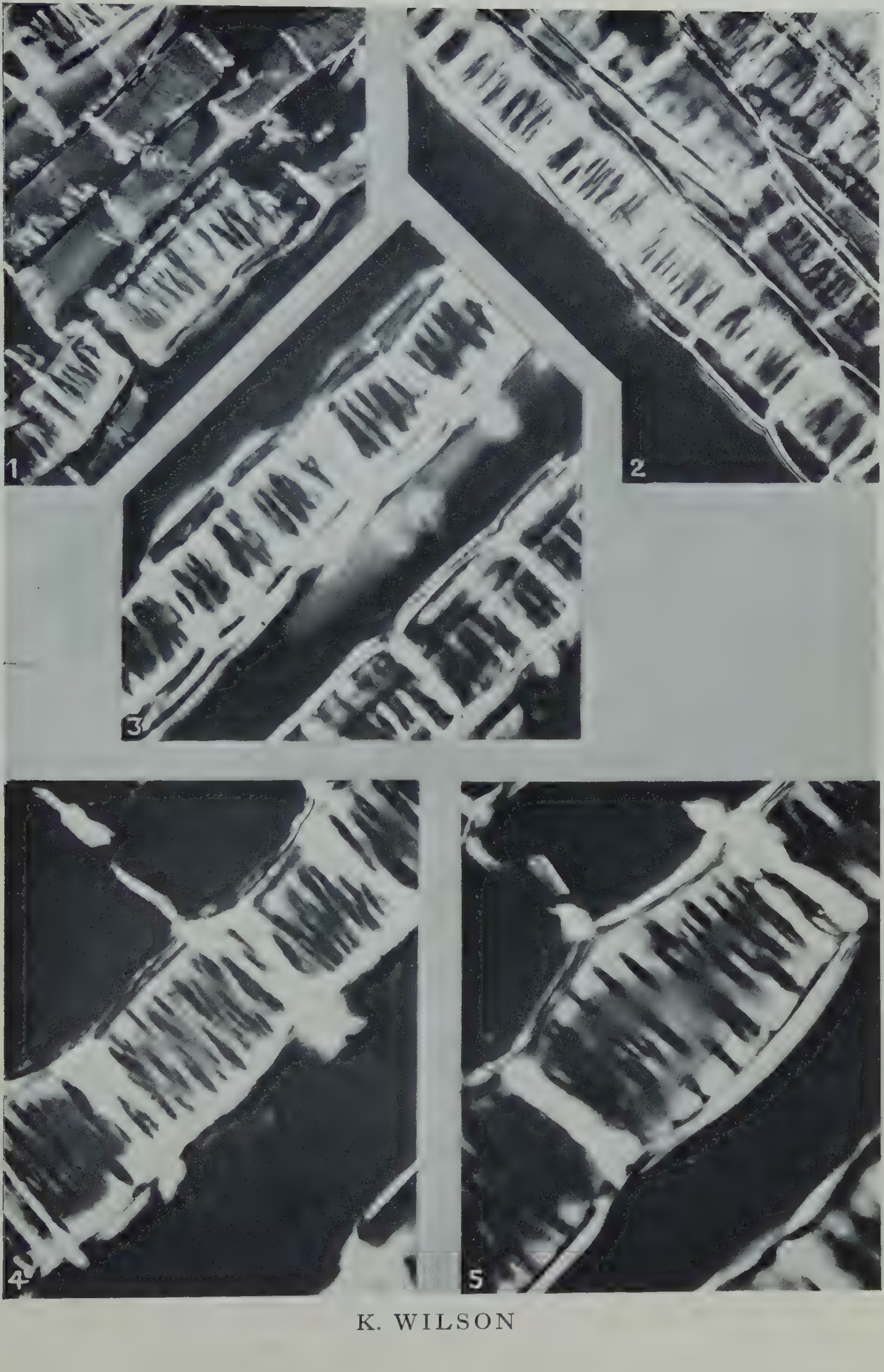
EXPLANATION OF PLATE

Hippuris vulgaris. Longitudinal walls between cortical cells, from internodes of various lengths, as seen in face view, to show pit-fields. Polarization photomicrographs, ×600. The internode lengths are: Fig. 1, 0·36 mm., Fig. 2, 0·6 mm., Fig. 3, 1·1 mm., Fig. 4, 3·5 mm., and Fig. 5, 9·6 mm.

The longitudinal direction lies obliquely to the length of the page. The dark areas between files of cells are intercellular spaces, and the wide transverse white bands are horizontal cell-walls seen in section. These appear much too thick in the photographs as a result of halation arising from the excessive brilliance of their images.



K. WILSON

A Population Study of *Spergula arvensis*

I. Two Clines and their Significance

BY

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(*University College, London*[1])

With one Plate and eight Figures in the Text

Abstract

Two seed-coat forms (the presence or absence of papillae) and two hairiness forms (medium or densely hairy) of *Spergula arvensis* are differently distributed through the British Isles, their relative frequencies being correlated with latitude and altitude. The proportions of the non-papillate and densely hairy forms increase with increasing distance from Kent to the north-north-west and also with increasing altitude.

A possible explanation is that the diverse forms indicate selective differences. Experiments have been carried out to test this hypothesis and the evidence supports it. The non-papillate form produces a lower proportion of fertile capsules than the papillate form when grown at a high temperature and low humidity. Also the non-papillate seeds germinate more readily than the papillate seeds at low temperatures, whereas at higher temperatures the reverse is true.

It is suggested that hybrid vigour, fluctuating selection intensities, and migration may all play a part in maintaining the observed distribution of the various forms.

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INTRODUCTION

WHERE two or more distinct forms are present within a species, quantitative data about their distribution often show them to be differently distributed and this can sometimes be correlated with environmental factors. Such correlations suggest possible selective advantages of the

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[Annals of Botany, N.S. Vol. 22, No. 88, Oct. 1958.]

forms but it needs experimental evidence to prove this conclusively. Correlations of this type have been observed in a few plants and many animal species. Among the plants the situation is most completely worked out for two forms of *Ricinis communis* (Harland, 1947). The forms differ in the presence or absence of a waxy bloom on the stems, and Harland has shown that in Peru, where it grows as a weed, the proportion of the waxy form increases with altitude. Increasing altitude is correlated with increasing sunlight and decreasing fog, and the waxy form has been shown to be at a selective disadvantage at low altitudes due to its much reduced ability to fruit in sunless foggy areas. Other examples of ratio-clines have been described for *Trifolium repens* (Daday, 1954), in *Diervilla lonicera* (Fassett, 1942) and in *Teucreum canadense* (McLintock and Epling, 1946). Huxley (1955) refers to similar work done with many animal species.

Spergula arvensis has two forms of seedcoat, one with and the other without papillae (see Plate) and also two degrees of hairiness of stem and leaves, either medium or densely hairy. The inheritance of these characters will be considered in a later paper. Nicholson (1880) examined herbarium specimens and county records of *S. arvensis* and his observations suggested that the papillate-seeded form is restricted to the southern part of the British Isles and to the warmer parts of the world, whereas the non-papillate form is mostly restricted to the colder regions. Both he and Druce (1891) noted from observations of fresh material that non-papillate seedcoats were often associated with dense viscid hairiness of stems and that papillate seeds were often associated with medium hairiness. Druce pointed out that this wa snot invariably the case. Boenninghausen (1824) had given these associations specific status, naming the former *S. vulgaris* and the latter *S. sativa.* Mertens and Koch (1831) gave the associations varietal status, but Clapham (1952) considers that there is no justification for their separate recognition and I agree for reasons I shall discuss later.

In the first part of this paper I shall describe the results of a more detailed study of the distribution and relation of the seedcoat and hairiness characters. Correlations between the distributions and climatic factors will be noted. The second part consists of the results of some experiments designed to test whether these climatic factors act as selective agents.

A. DISTRIBUTION OF SEEDCOAT AND HAIRINESS CHARACTERS

1. *Sampling methods*

S. arvensis is a common weed of arable land, particularly on acid, sandy soils. In each case I have taken the *S. arvensis* plants in one field as the population to be sampled. One might expect a reasonable degree of uniformity of habitat within one field while the differences may well be much greater between fields owing to diversity of methods of cultivation, fertilizers, cropping, &c. Observations have been made in twenty-two localities in the British Isles and I have sampled several fields in each locality wherever possible.

I picked up plants at approximately 5-m. intervals round the edges of the fields and scored them individually for both seedcoat and hairiness characters. The seedcoats have either a very large number of papillae or none at all. A very few intermediates were found, and these were classified as such. Plants were classified as densely hairy if they had an average of greater than 20 hairs per 1·5 cm. as seen in silhouette on the lowest internode. Most plants fell easily into one of these two groups and had either about 5 hairs or 40 per 1·5 cm. A sample size of 50 plants for each field was considered to be large enough for statistical tests to be applied to the observed proportions and this was obtained wherever possible. However, in many fields it was impossible to obtain a sample as large as 50. Samples of under 10 plants per field have all been ignored in the following account. In each field sampled, observations were also made on crop, aspect, slope, soil, and a soil sample was collected for the determination of pH.

Herbarium material at Kew and the British Museum was also examined and scored for seedcoat character. It was impossible to determine hairiness from dried specimens. The scoring of hairiness in the field is sometimes made very difficult by the fact that the hairs wither and lie flat against the stems as the plants grow old.

In presenting the results I shall consider first the seedcoat character, second hairiness, and thirdly the relation between the two.

2. *Seedcoat Character*

(*a*) *Variation with season.* The bulk of the observations were made between mid-July and mid-September 1955 and 1956. Before the results of these observations could be interpreted, the first question to be answered was whether the proportions of the two types vary from one time of the year to another. Observations were made on three fields on the Surrey–Kent border in mid-July (the earliest time mature seeds could be found) and again in late September. There was no significant difference between the July and September samples. ($\chi^2 = 0{\cdot}0313$, d.f. $= 1$, $p \simeq 0{\cdot}86$.)

(*b*) *Variation from year to year.* Samples were also taken from the same fields in two localities (Surrey and Cheshire) in two consecutive years to see whether there were any changes from year to year. The results are given in Tables 1 and 2.

The calculation of χ^2 on the totals gives $p = 0{\cdot}48$ for Surrey and there is therefore no evidence that the proportion of the two types varies from year to year. For Cheshire $p = 0{\cdot}03$ and the results are therefore rather inconclusive although they suggest an increase in the proportion of papillate-seeded plants. It should be noted that there is no systematic change for each field, in some fields (1, 3, 4, and 5) there is an increase and in others (2 and 6) there is a decrease.

The summer of 1955 was unusually hot and dry in July and August, and therefore these data also show that a single year of unusual climatic conditions does not markedly affect the proportions of papillate- and non-papillate-seeded plants.

It will be seen from these tables that there is a variation from field to field ($p<0·001$) and I shall consider this point later.

Tables Showing the Change in Proportions from July 1955 to July 1956

TABLE 1

SURREY

		July 1955		July 1956	
Field	Seedcoat	Number	Prop.	Number	Prop.
1	Pap.	43	0·248	32	0·302
	Non-pap.	130	0·752	74	0·698
2	Pap.	61	0·410	20	0·444
	Non-pap.	88	0·590	25	0·556
3	Pap.	20	0·191	24	0·270
	Non-pap.	85	0·809	65	0·730
Total	Pap.	124	0·290	76	0·317
	Non-pap.	303	0·710	164	0·683

$\chi^2 = 0·505 \quad p \simeq 0·48$

TABLE 2

CHESHIRE

		July 1955		July 1956	
Field	Seedcoat	Number	Prop.	Number	Prop.
1	Pap.	17	0·450	18	0·462
	Non-pap.	21	0·550	21	0·538
2	Pap.	13·5*	0·246	3	0·120
	Non-pap.	41·5	0·754	22	0·880
3	Pap.	3	0·058	3	0·271
	Non-pap.	48	0·942	8	0·729
4	Pap.	3	0·055	5·5*	0·333
	Non-pap.	52	0·955	15·5	0·667
5	Pap.	14	0·186	15	0·396
	Non-pap.	68	0·816	23	0·604
6	Pap.	19	0·317	8	0·195
	Non-pap.	41	0·683	33	0·805
Total	Pap.	66·5	0·210	52·5	0·300
	Non-pap.	271·5	0·790	122·5	0·700

$\chi^2 = 4·89 \quad p \simeq 0·03$

* Intermediates were classed as 0·5 papillate and 0·5 non-papillate because they were shown to be hybrids.

(*c*) *Distribution throughout the British Isles.* The locality proportions have been calculated from the total numbers of the two types of plant from all the fields in the locality, rather than as an average of the proportions calculated for each field. This gives weight to the sample size which varies between 10 and 170. The usual procedure of weighting each sample proportion according to the inverse of the sample variance cannot be followed, as in the cases where the plants are all of one type the variance is, of course, zero. The results of samples made in various localities throughout the British Isles are represented in

Fig. 1. The black segment of each circle represents the proportion of papillate-seeded plants in the locality and the white segment represents the proportion of non-papillate-seeded plants. It will be seen that there is an increase in the proportion of non-papillate-seeded plants with increasing distance north and also, but less obviously, with increasing distance west. Using the angular transformation, the calculation of the correlation coefficient between the proportion of non-papillate and distance north gives a value of $r = 0{\cdot}828$ ($p < 0{\cdot}001$) and between the proportion of non-papillate and distance west gives $r = 0{\cdot}530$ ($p < 0{\cdot}01$). The highest value of r is obtained between the proportion of non-papillate and distance north-north-west (NNW.). In this case $r = 0{\cdot}936$. The proportion of non-papillate is plotted against distance NNW. on Fig. 2.

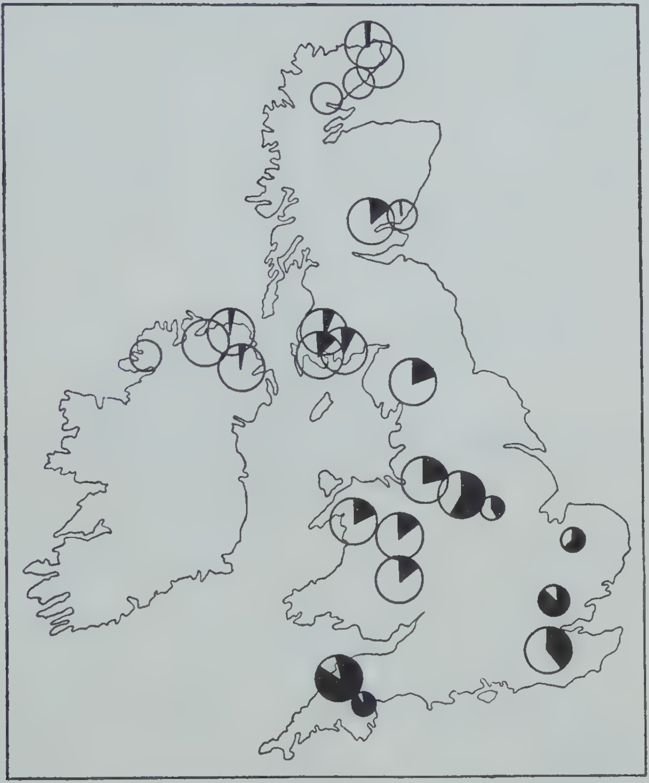

FIG. 1. Map showing the proportions of non-papillate- and papillate-seeded plants of *S. arvense* in different localities.

White segments = proportions of non-papillate seeds; black segments = proportion of papillate seeds; large circles—100 plants or more; medium circles—50 to 100 plants, small circles—10 to 50 plants.

I have examined the possibility that this distribution is correlated with some climatic factor. There is no close correlation with any single factor of temperature or rainfall, but there is a better correlation with mean summer temperature than any other. This relation is shown on Fig. 3. There is no

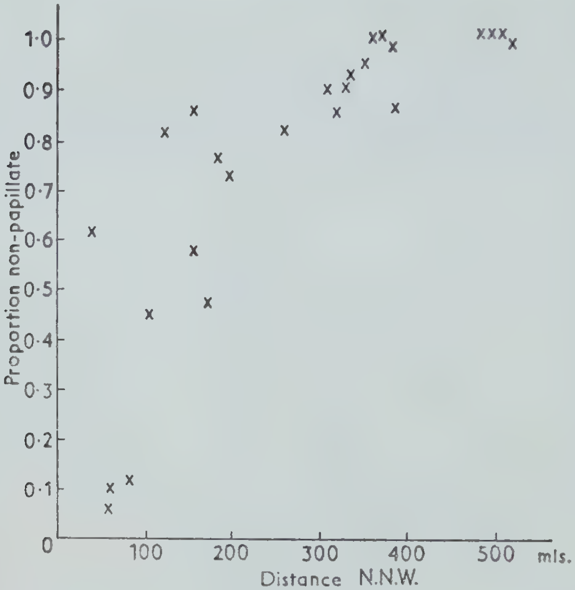

FIG. 2. Graph showing the correlation between the proportion of non-papillate-seeded plants and the distance NNW. (in miles from Dover).

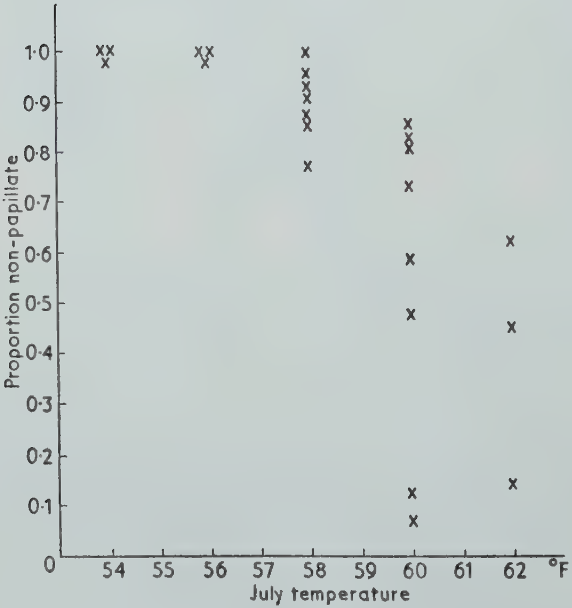

FIG. 3. Graph showing the correlation between the proportion of non-papillate-seeded plants and the July temperature.

close relation between the observed cline and winter temperature, winter or summer rainfall.

(*d*) *Distribution throughout Europe.* Although in herbarium collections a locality is usually represented by only one specimen of a species, interesting information can be gained from these presence and absence data.

I examined the collections at Kew and the British Museum, and the results are represented on Fig. 4. The same general trend of north-north-west to south-south-east decrease in non-papillate plants which is seen in the British Isles is present throughout Europe. Again, the correlation with summer temperature is closer than with any other single factor. Winter temperature increases from north-east to south-west. Summer rainfall does not show any marked trend throughout Europe and winter rainfall increases from east to west.

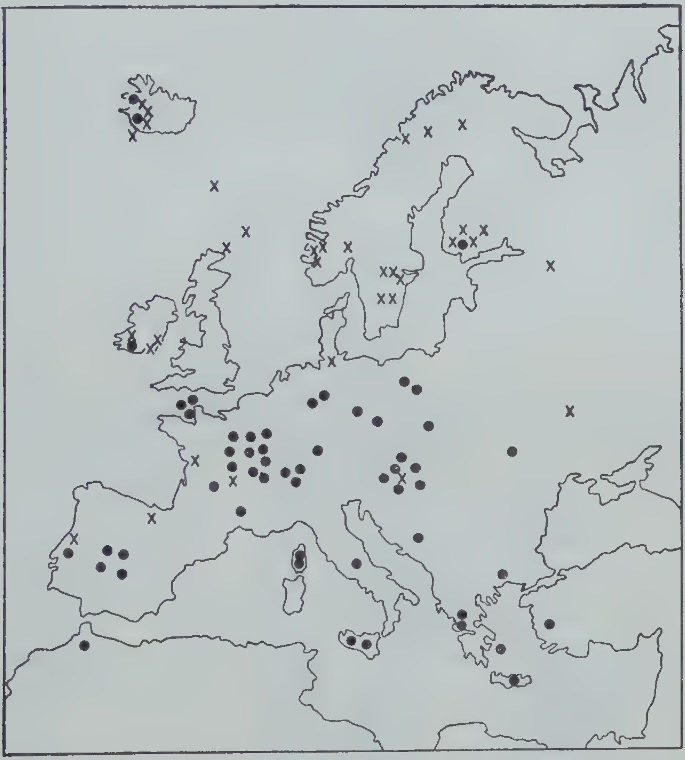

FIG. 4. Map showing the distribution of the papillate and non-papillate forms throughout Europe.

● = papillate
× = non-papillate

(*e*) *Distribution with altitude.* From the point of view of the climate, an increasing height above sea-level is equivalent to increasing latitude. When the existence of the cline with latitude was apparent, I made observations to see whether there was also a cline with altitude. Since *S. arvensis* is restricted to arable fields it is not possible to get very great ranges of height within any locality in the British Isles. However, a range of about 700 ft. was found in Shropshire and one of 650 ft. in Derbyshire. The results are given in Table 3. There is a significant increase in the proportion of non-papillate plants with increasing height ($p = 0{\cdot}003$ for Shropshire and $p < 0{\cdot}001$ for Derbyshire).

A height of 300 ft. is not sufficient to show this. Of four localities where a range of 300 ft. was found, two (Herefordshire and Galloway) showed an increase in non-papillate with height, one (Westmorland) an increase of papillate with height and one (Devon) no trend at all.

TABLE 3

Showing the Relation between the Proportion of Non-papillate-seeded Plants and Altitude

Locality	Alt. in feet.	No. of fields	No. pap.	No. non-pap.	Prop. non-pap.
Derbyshire	350	2	29	5	0·147
	850	3	43	29	0·403
	1,000	3	44	73	0·622
Shropshire	625	5	36	155	0·811
	1,350	5	23·5	217·5	0·902

TABLE 4

Showing the Change in Proportions from July 1955 to July 1956 in Cheshire

		July 1955		July 1956	
Field	Hairiness	Number	Prop.	Number	Prop.
1	Dense	12	0·316	17	0·436
	Medium	26	0·684	22	0·564
2	Dense	7	0·127	0	0
	Medium	48	0·873	25	1·000
3	Dense	8	0·157	1	0·091
	Medium	43	0·843	10	0·909
4	Dense	9	0·173	2	0·095
	Medium	43	0·827	19	0·905
5	Dense	28	0·342	11	0·289
	Medium	54	0·658	27	0·711
6	Dense	41	0·684	2	0·049
	Medium	19	0·316	39	0·951
Total	Dense	105	0·311	33	0·217
	Medium	233	0·689	142	0·783

$\chi^2 = 8{\cdot}739 \quad p \simeq 0{\cdot}005$

(*f*) *Variations within a locality.* It can be seen from the examples in Table 1 that there is a considerable difference from field to field in the proportions of the two forms within a locality. The same is true for all localities where the proportion of neither form is below about 0·1. I have looked for correlations between the proportions and soil texture, soil pH, aspect of field and crop, and have observed none. A possible alternative explanation is that seeds from distant localities with different proportions have been introduced as contaminants of agricultural seed. This is a known method of dispersal for *S. arvensis* (see Broad, 1952).

3. *Hairiness Character*

The results are presented under the same scheme of headings as the results for the seedcoat character. There are, however, larger gaps in the picture because of the difficulties mentioned before—the hairs lie flat against the stem as the plants grow old and it is impossible to determine hairiness from dried specimens.

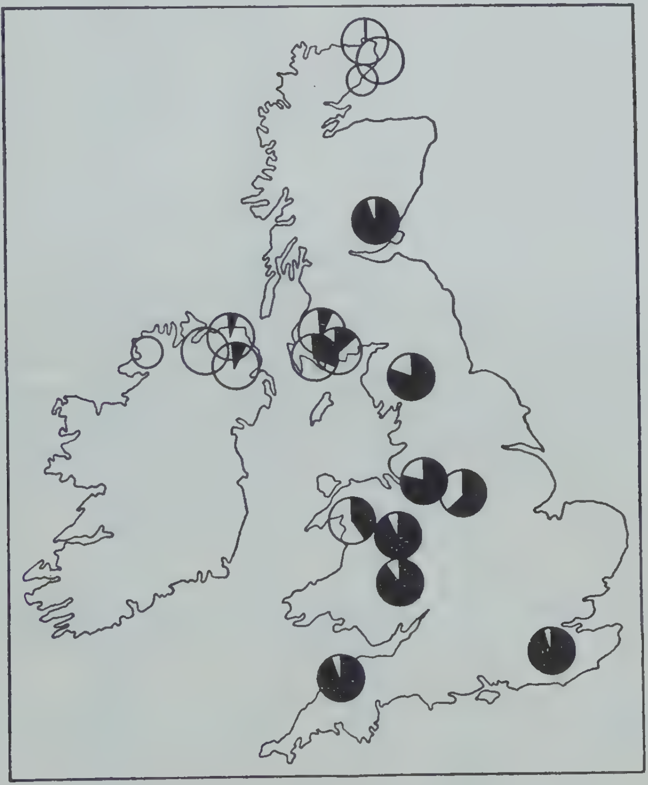

FIG. 5. Map showing the proportion of densely hairy and medium-hairy plants.

White segments = proportion of densely hairy plants; black segments = proportion of medium-hairy plants; large circles—100 plants or more, medium circles—50 to 100 plants.

(*a*) *Variation with season.* Again, observations were made on three fields on the Surrey–Kent border in mid-July and in late September. There was no significant difference between samples taken at different seasons ($p = 0{\cdot}20$).

(*b*) *Variation from year to year.* The results for Cheshire (see Table 4) show that there is a significant decrease in the total proportion of densely

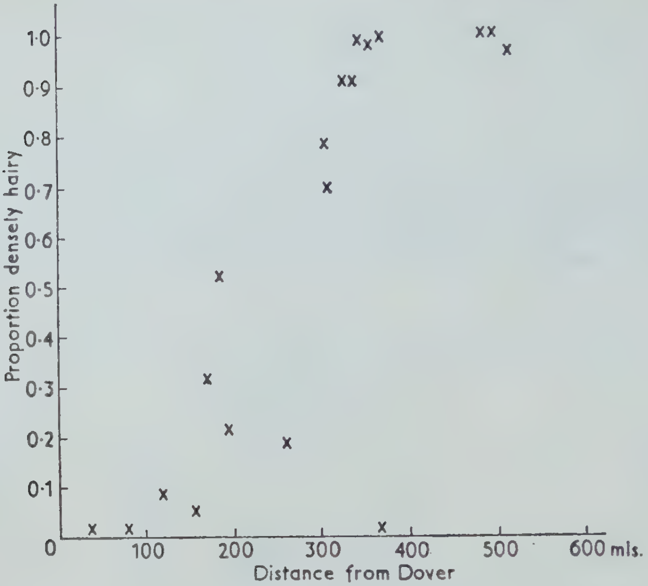

FIG. 6. Graph showing the correlation between the proportion of densely hairy plants and the distance NNW. (in miles from Dover).

hairy plants from 1955 to 1956 ($p \simeq 0{\cdot}005$). It can be seen from closer examination of the table that this is due to a decrease in all except one of the fields. It appears that, contrary to the results for seedcoat, a change in proportion can take place in a single year. As was noted before, July and August 1955 were exceptionally warm and dry and this may be supposed to have an effect similar to that of increased distance south and east.

TABLE 5

Showing the Relation between the Proportion of Densely Hairy Plants and Altitude

Locality	Alt. in feet	No. of fields	No. dense hairy	No. medium hairy	Proportion dense-hairy
Derbyshire	350	2	4	30	0·118
	850	3	24	51	0·292
	1,000	2	34	47	0·420
Shropshire	625	5	0	180	0
	1,350	5	18	223	0·075

This is the only locality for which results were obtained.

(*c*) *Distribution throughout the British Isles.* In general the proportion of densely hairy plants increases with increasing distance north and west. Both the Shropshire and Perthshire populations have proportions too low to fit this picture well. The results are expressed on Figs. 5 and 6.

(*d*) *Variation with altitude.* In both Derbyshire and Shropshire there is a significant increase in the proportion of densely hairy plants with increasing altitude ($p < 0·001$ in both cases). The results are given in Table 5. The rate of increase with height is much less in Shropshire than Derbyshire.

4. *Distributional Correlations between Seedcoat and Hairiness Characters*

There is a rough correlation between the proportion of non-papillate-seeded plants and the proportion of densely hairy plants throughout the country (see

TABLE 6

		Hairiness				
Locality	Seedcoat	Medium	Dense	Total	With Yate's corr.	Approx. p.
Surrey	Pap.	128	1	129		
	Non-pap.	265	10	275		
	Total	393	11	404	1·7148	0·21
Devon	Pap.	213	3	216		
	Non-pap.	27	3	30		
	Total	240	6	246	4·9884	0·04
Herefordshire	Pap.	37	2	39		
	Non-p.	99	11	110		
	Total	136	13	149	0·3553	0·60
Derbyshire	Pap.	75	26	101		
	Non-pap.	48	36	86		
	Total	123	64	187	7·0147	0·01
Shropshire	Pap.	57·5*	2	59·5		
	Non-pap.	356·5	16	372·5		
	Total	414	18	432	0·0002	0·99
Cheshire '55	Pap.	92	37	129		
	Non-pap.	292	99	391		
	Total	384	136	520	0·5672	0·49
Cheshire '56	Pap.	50	10	60		
	Non-pap.	129	22	151		
	Total	179	32	211	0·0136	0·91
Merionethshire	Pap.	32	33	65		
	Non-pap.	75	103	178		
	Total	107	136	243	4·777	0·04
Westmorland	Pap.	34	3·5*	37·5		
	Non-pap.	125	37·5	162·5		
	Total	159	41	200	2·739	0·10
Wigtownshire	Pap.	17	14	31		
	Non-pap.	36	110	146		
	Total	53	124	177	11·103	0·001
Kirkcudbright-shire. a.	Pap.	9	2	11		
	Non-pap.	15	89	104		
	Total	24	91	115	23·426	0·001
,, b.	Pap.	3	8	11		
	Non-pap.	9	108	117		
	Total	12	116	128	2·525	0·11
Perthshire	Pap.	50·5	1	51·5		
	Non-pap.	253·5	4	257·5		
	Total	204	5	309	0.649	0·55
Northern Ireland	Pap.	4	18	22		
	Non-pap.	15	841	856		
	Total	19	859	878	20·136	0·001
Caithness	Pap.	1	1	2		
	Non-pap.	2	90	92		
	Total	3	91	94	3·145	0·08

* Intermediates were classed as 0·5 papillate and 0·5 non-papillate because they were later shown to be hybrids.

Fig. 7). Using the angular transformation of the proportions, the correlation coefficient $r = 0{\cdot}762$. For this value of r, $p = 0{\cdot}001$. The significance of this will be discussed later.

It is interesting to note that in eleven localities medium hairiness is evenly distributed between plants with papillate seeds and plants with non-papillate seeds (see Table 6). This must mean that there is no genetic association between the two characters. In three other localities (Northern Ireland, Wigtownshire, and Kirkcudbrightshire) there is a significant, although by no means unfailing, association between medium hairiness and papillate seeds and between dense hairiness and non-papillate seeds. The apparent association in Derbyshire is solely due to altitudinal effects—there is no association if the calculations at the different altitudes are made separately ($\chi^2 = 5{\cdot}531$, $p \simeq 0{\cdot}25$).

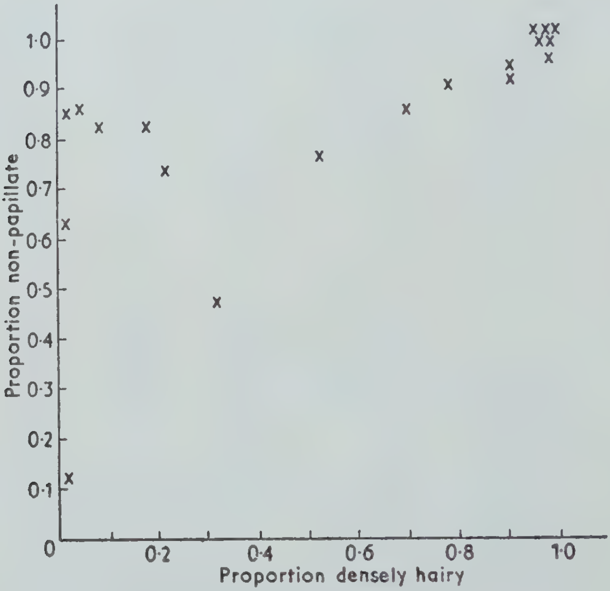

FIG. 7. Graph showing the distributional correlation between the seedcoat and hairiness characters.

B. EXPERIMENTAL EVIDENCE FOR SELECTION

As will be discussed in more detail later, the two main types of explanation of clines are in terms of migration or of selection. It is possible to test hypotheses of migration only over decades, whereas the possibility exists of obtaining results from experiments to test selection in as little as a single generation. It should be noted that a negative result would be inconclusive since it could mean that the effects of selection might be too small to be observable in one generation but which might be additive and important in nature.

Since there is a rough correlation between the distribution of the forms and summer temperature, the first obvious factor to test was the effect of temperature on the growth of the plant. As the correlation was not close it seemed probable that other factors were also involved and the effect of humidity on the growth of the plant, the effect of temperature on germination, and the effect of temperature on seed during the dormant phase were also tested.

Different combinations of hairiness and seedcoat characters were tested in order to see which were the most affected by the various conditions.

1. *The Effect of Temperature and Humidity on the Growth of* S. arvensis

Material and methods. Seeds were collected from various localities in the British Isles, and seeds from each plant were packeted separately. Since about 97 per cent. of the breeding in nature is selfing, sibs are genetically very similar. Sibs were used for comparison under different treatments.

The seeds were germinated under the fluctuating temperature and humidity conditions of the greenhouse and were later transferred to the test conditions. The plants were grown one in a pot and there was no competition for light, nutrients, or water.

Experiment 1. In the first experiment, using material from Northern Ireland, seedlings were put into conditions of high temperature (21° C.) and low humidity (40 per cent. relative humidity) when they were about 6 cm. high with two nodes. They were given 16 hours of light at 700 f.c. (from mercury vapour and incandescent lamps) succeeded by 8 hour-dark periods. The experiment was duplicated and controls were grown in the greenhouse with daylight (March and April), temperature 13–16° C. and humidity about 70 per cent. Seedlings produced from a papillate-seeded medium-hairy parent and seedlings from a non-papillate densely hairy parent were used.

After about a week at high temperature and low humidity it became clear that the leaves of the non-papillate densely hairy plants were wilting and dying back from the tips, whereas the medium-hairy papillate plants were quite normal. (As it is usually supposed that the presence of hairs reduces water-loss this cannot be a simple matter of water relations.) Both types in the control conditions were growing normally.

After 5 weeks all 8 densely hairy non-papillate plants had either died or were extremely weak, whereas only 1 out of 6 of the medium-hairy papillate plants had died and the others had produced seeds. Both types in the control had grown normally and produced seeds. These results are expressed in Table 7 as average dry weights of shoot per plant after 5 weeks.

When this experiment was carried out, facilities were not available for raising the humidities above 40 per cent. r.h. but in all further experiments the lowest humidity used was 55 per cent. r.h. This was done because 55 per cent. falls within the range found in the British Isles, whereas 40 per cent. does not.

TABLE 7

Average Dry Weights of Shoots under Different Conditions of Temperature and Relative Humidity (r.h.)

	Conditions		
Material	A. 21° C. 40% r.h.	B. 21° C. 40% r.h.	C. 13–16° C. c. 70% r.h.
Northern Ireland. Non-papillate, densely hairy	0·018 g. (4 plants)	0·047 g. (4 plants)	1·639 g. (4 plants)
Northern Ireland. Papillate, medium hairy	0·417 g. (3 plants)	0·212 g. (3 plants)	1·975 g. (2 plants)

A and B are duplicates.

Experiment 2. The question which arises from this result is whether the operator factor is temperature or humidity. An experiment using material from the same source was carried out at:

(*a*) 21° C. and 55 per cent. r.h.
(*b*) 21° C. and 75 per cent. r.h.
(*c*) 16° C. and 55 per cent. r.h.
(*d*) 16° C. and 75 per cent. r.h.
(*e*) fluctuating in greenhouse.

The temperature on hot days sometimes rose above those given, but the night temperatures and temperatures most of the day were those given. In each case daylight was the light source and day-length was about 16 hours. The seedlings were transferred to the experimental conditions when they had about 4 leaves at the first node.

TABLE 8

Proportion of Fertile Capsules under Different Conditions of Temperature and Relative Humidity (r.h.)

		Conditions				
Material		21° C. 55% r.h.	21° C. 75% r.h.	16° C. 55% r.h.	16° C. 75% r.h.	Control
Northern Ireland. Non-papillate, densely hairy	Prop. fertile capsules	0·720	0·872	1·000	1·000	1·000
	Variance	0·0142	0·0165	0	0	0
	No. plants	5	4	4	4	4
Northern Ireland. Papillate, medium hairy	Prop. fertile capsules	1·000	1·000	1·000	—	1·000
	Variance	0	0	0	—	0
	No. plants	4	2	1	—	3

In this experiment there was no significant difference in the dry weight of shoot produced. There was, however, a difference in the proportion of fertile capsules produced. The non-papillate densely hairy plants showed a reduction in fertility at the high temperatures which was particularly marked at low humidities. In contrast, all the capsules of the papillate medium-hairy plants were fertile under all the five conditions tested. See Table 8. The difference between the two humidities at 21° C. is significant as can be seen from Table 9.

The conclusion is that temperature is the more important factor, but that its effect is more pronounced at low humidities.

Experiment 3. The experiment described next was designed to test whether the low fertility is associated with the seedcoat or hairiness character and also to test whether the results using material collected in Northern Ireland could be extended to material collected in other localities. The results are given in Table 10.

TABLE 9

Numbers of Fertile and Non-fertile Capsules on Non-papillate, Densely Hairy Plants from Northern Ireland

	55% r.h. 21° C.	75% r.h. 21° C.	Totals
Fertile	55	71	126
Non-fertile	19	6	25
Totals	74	77	151

$\chi^2 = 8{\cdot}7365$; $p \simeq 0{\cdot}003$

TABLE 10

Average Proportions of Fertile Capsules on Plants Grown under Different Conditions of Temperature and Humidity

Material		Conditions				
		21° C. 55% r.h.	21° C. 75% r.h.	16° C. 55% r.h.	16° C. 75% r.h.	Control
Caithness						
Non-pap., dense hairs	Prop. fertile capsules	0·061	0·533	1·000	0·933	—
	Variance	0·004	0·146	0	0·014	—
	No. plants	3	3	3	3	—
Pap., dense hairs	Prop. fertile capsules	0·922	0·553	0·993	1·000	1·000
	Variance	0·004	0·103	0·003	0	0
	No. plants	3	3	4	4	4
Cheshire						
Non-pap., medium hairy	Prop. fertile capsules	0·500	0·516	1·000	0·895	0·822
	Variance	0	0·001	0	0	0·050
	No. plants	1	2	2	1	2
Pap., medium hairy	Prop. fertile capsules	1·000	1·000	1·000	1·000	1·000
	Variance	0	0	0	0	0
	No. plants	1	2	1	2	1
Wigtownshire						
Non-pap., dense hairs	Prop. fertile capsules	1·000	0·960	1·000	1·000	—
	Variance	0	0	0	0	—
	No. plants	1	1	1	1	—
Pap., medium hairy	Prop. fertile capsules	0·800	0·800	1·000	1·000	—
	Variance	0	0·040	0	0	—
	No. plants	1	2	2	2	—

966.88

An analysis of the Caithness and Cheshire material grown at 21° C. shows that there is a significant difference between non-papillate- and papillate-seeded plants, but that there is no significant difference between medium and densely hairy ones. It would be preferable to compare the four different combinations of the two characters in material collected from the same locality, but this has not been possible so far. The results from the Wigtownshire material do not fit the picture well and will be discussed later.

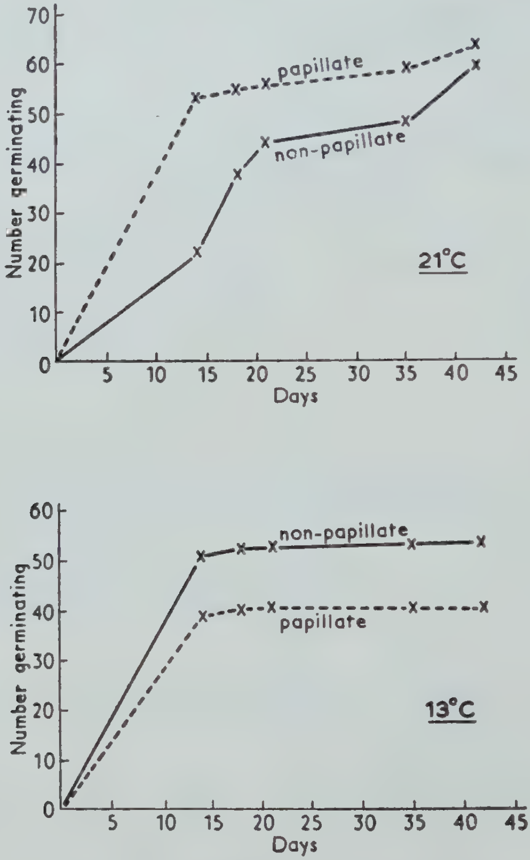

FIG. 8. Graphs showing the rates of germination of papillate and non-papillate seeds at different temperatures.

2. *The effect of temperature on germination*

Seeds collected in Cumberland were put to germinate on moist sand at 21° C. and 13° C.—100 papillate seeds and 100 non-papillate seeds at each temperature.

At 21° C. the non-papillate seeds were slower in germinating than the papillate seeds and at 13° C. the opposite was found, the papillate were slower than the non-papillate. After 40 days the number of germinated seeds at 21° C. was almost the same with both types of seed, but after the same period at 13° C. more non-papillate than papillate seeds had germinated. (See Fig. 8.)

An attempt was made to repeat this experiment with material collected from Perth and from Westmorland, but both types from each locality almost completely failed to germinate for some quite unknown reason.

3. *The Effect of Soil Temperature during Dormancy on Germination*

For this experiment both types of seeds collected in Cumberland and in Surrey were stored, some at 4° C. and some at 16° C. for 3 months in moist soil. There were, however, no differences in germination capacity between papillate and non-papillate seeds at the end of this period.

DISCUSSION

The results show that there are ratio-clines in the seedcoat and hairiness characters of *S. arvensis* which extend in a NNW.–SSE. direction across the British Isles and probably across the whole of Europe as well. Haldane (1948) has pointed out that there are two possible types of explanation of a ratio-cline depending on whether the situation is a transient or a permanent one. A cline may be a stage in the migration of a gene from a centre of its origin or introduction, or on the other hand it may be due to the fact that one form is favoured in one part of the habitat, the other in another part.

As I mentioned before, an increase in altitude is associated with climatic changes similar to those produced by an increase in latitude. The clines, both for seedcoat and hairiness, are found to exist with altitude as well as latitude and this very strongly suggests that climatic factors are controlling the distribution of the various forms. This provides good evidence that selection and not migration is the relevant explanation of the clines.

The results of the cultivation experiments also provide evidence to support the selection hypothesis. The production of infertile capsules is associated with the seedcoat character rather than the hairiness character and it appears to be due to a failure of the pollen to mature. Only small yellowish-green anthers are produced instead of the usually larger bright yellow ones. Since this effect cannot conceivably be due to the structure of the ovule integuments (which later become the seedcoat), it must be due to a pleiotropic effect of the gene controlling this character or due to a linked gene. It is just possible that the anomalous results found in the Wigtownshire material are due to the separation of a linkage.

It should be noted that the experiments on fertility show that the non-papillate form has a selective disadvantage in high temperatures (and therefore in southerly regions) but they do not show any disadvantage of the papillate form in the north. For the maintenance of a cline it is important that both forms should be at a disadvantage in different regions. If this were not so, it would be expected that the form disadvantageous in one region would be gradually superseded not only in that region but throughout its range, due to the effects of long-distance dispersal. Any plants which were the result of migration into the disadvantageous region would be selected against, but there would be no compensating effect in the reverse direction.

It is possible that the temperatures used were not low enough to show a disadvantage of the papillate type, but attempts to grow plants at low (10°–13° C.) temperatures were unsuccessful for both types.

One disadvantage of the papillate form in the north is suggested by the experiment on the rate of germination at different temperatures. The papillate seeds germinate more slowly and to a lower final proportion than the non-papillate seeds at low temperatures (13° C.). This experiment suggests another disadvantage of the non-papillate form in the south, for at high temperatures (21° C.) the non-papillate seeds germinate more slowly than the papillate seeds. Since *S. arvensis* plants continue to produce seeds until they are killed by harvesting operations or autumn frosts, late germination and late maturity reduces the number of seeds produced and also reduces the possibility of more than one generation in a season, I suggest that the selective advantage of early germination lies in this. It is conceivable that the form of the seedcoat itself may affect germination, and so it is possible that this is a case of a direct effect of the genes controlling the presence or absence of papillae rather than a linkage or pleiotropic effect.

It seems unlikely that these two types of experiment reveal the only selective differences between the two seedcoat forms and they have revealed no differences between the hairiness forms. The initial experiment, where the non-papillate, densely hairy plants grew so badly at high temperatures and low humidity, indicated that there are physiological effects other than on fertility and germination. I think it is most probable that further experiments and observations would show other differences between the various forms, both of seedcoat and hairiness.

One of the interesting problems which now arises is that of how the cline is maintained. How is it that two forms of the same species continue to grow side by side in some localities when, as Fisher has shown, only a very slight selective advantage of one form would be expected in time to suppress completely the other form?

In some cases where two forms grow together there is good reason to believe that the hybrid has a selective advantage and there are several examples known of this (e.g. sickle-cell anaemia—see Allison, 1955). Precise information on the selective advantages of the homozygous and heterozygous forms under natural conditions are necessary to decide this. However, it is very unlikely to play a large part in the explanation of the *S. arvensis* case since, as will be shown in a later paper, there is a high percentage of selfing in the species and few hybrids are produced.

Another possibility is that the selective forces fluctuate, favouring each of the two forms alternately. There is some evidence to support this in the case of the hairiness character where two successive years of very different climate were associated with differences in the proportions of the two types (see p. 465). There is no good evidence in the case of the seedcoat character since there was no marked difference in the frequencies of the two forms in these years. There is, however, some discussion as to the effects of alternating selection coefficients. Dempster (1955) has shown that with strictly periodic changes a stable equilibrium may be maintained between forms and Kimura (1954) has shown that random oscillations result in quasifixation rather than a stable equilibrium. Crow (1955) believes that under the majority of circumstances likely to be encountered in nature, non-constant selective values will lead to fixation rather than maintenance of variability. Kimuru and others have pointed out in this connexion that changes in gene frequencies in response to fluctuating selections are singularly ineffective since they are always produced a generation too late.

The third possibility is that continual migration maintains the clines. This type of explanation seems more relevant to animal species because of their greater mobility (Haldane, 1948) and to plant species which are specially suited to long-distance dispersal of their seeds or pollen (e.g. wind pollination of *Pinus*—Langlet, 1936). However, the long-distance dispersal of *S. arvensis* seed with agricultural seed may be on a scale large enough to maintain the clines.

The correlation in the distribution of the proportion of non-papillate and the proportion of densely hairy plants provides some points of interest. Since in most localities these characters are not associated on individual plants, the probable explanation of the correlation is that both characters are selectively affected by the same factors of the environment. I suggest that the explanation of the three exceptional cases (Northern Ireland, Wigtownshire, and Kirkcudbrightshire), where there is association of papillate seeds and medium hairiness on individual plants, is that there has been recent long-distance dispersal from the south of England where plants are mostly papillate and medium hairy. I suggest that these plants have not been in the north long enough to be selected against or for crossing to have dissociated the two characters.

As I mentioned before (p. 3), several workers have given the associations of papillate seeds with medium hairiness and non-papillate seeds with dense hairiness particular taxonomic status. However, since in the large majority of localities there is no association of these characters, I conclude that there is no reason for doing so.

SUMMARY

1. A ratio-cline in phenotype frequency of a seedcoat character (presence or absence of papillae) is shown to exist, extending in a NNW.–SSE. direction across the British Isles. The proportion of plants with non-papillate seeds increases with distance NNW.

2. There is evidence that this cline extends in a similar direction across Europe.

3. A cline with altitude is described; the proportion of the non-papillate (northern) form increases with increasing altitude.

4. A cline in phenotype frequency of a hairiness character (densely or medium-hairy stems and leaves) is also described which extends in a similar direction across the British Isles and which shows a similar effect of altitude. In this case the proportion of densely hairy plants increases with increasing latitude and altitude.

5. In most localities there is no significant association of dense hairiness and non-papillate seeds or of medium hairiness and papillate seeds on individual plants. The conclusion is that these associations do not deserve taxonomic status.

6. Experiments show that (*a*) the non-papillate densely hairy plants wilt and die at high temperatures and low humidities, whereas papillate medium-hairy plants grow more normally under these conditions; (*b*) non-papillate seeded plants produce a lower proportion of fertile capsules than papillate seeded plants when grown at 21° C., and this effect is more marked at 55 per cent. relative humidity than at 75 per cent. r.h.; (*c*) non-papillate seeds germinate more rapidly than papillate seeds at 13° C., whereas at 21° C. the reverse is true.

7. These results suggest that the different distributions of the forms are environmentally determined.

8. The roles of migration, hybrid vigour, and fluctuating selection in the maintenance of the clines are discussed.

ACKNOWLEDGEMENTS

I should like to thank Professor W. H. Pearsall for his advice and encouragement during the course of this work. My thanks are also due to my husband, fellow research students, and members of the Ministry of Agriculture Advisory Service for help in various ways and to Mr. R. Teasdale for technical assistance. I am also grateful to the Department of Scientific and Industrial Research and the Central Research Fund of London University for financial support. The work described formed part of a Ph.D. thesis presented to the University of London.

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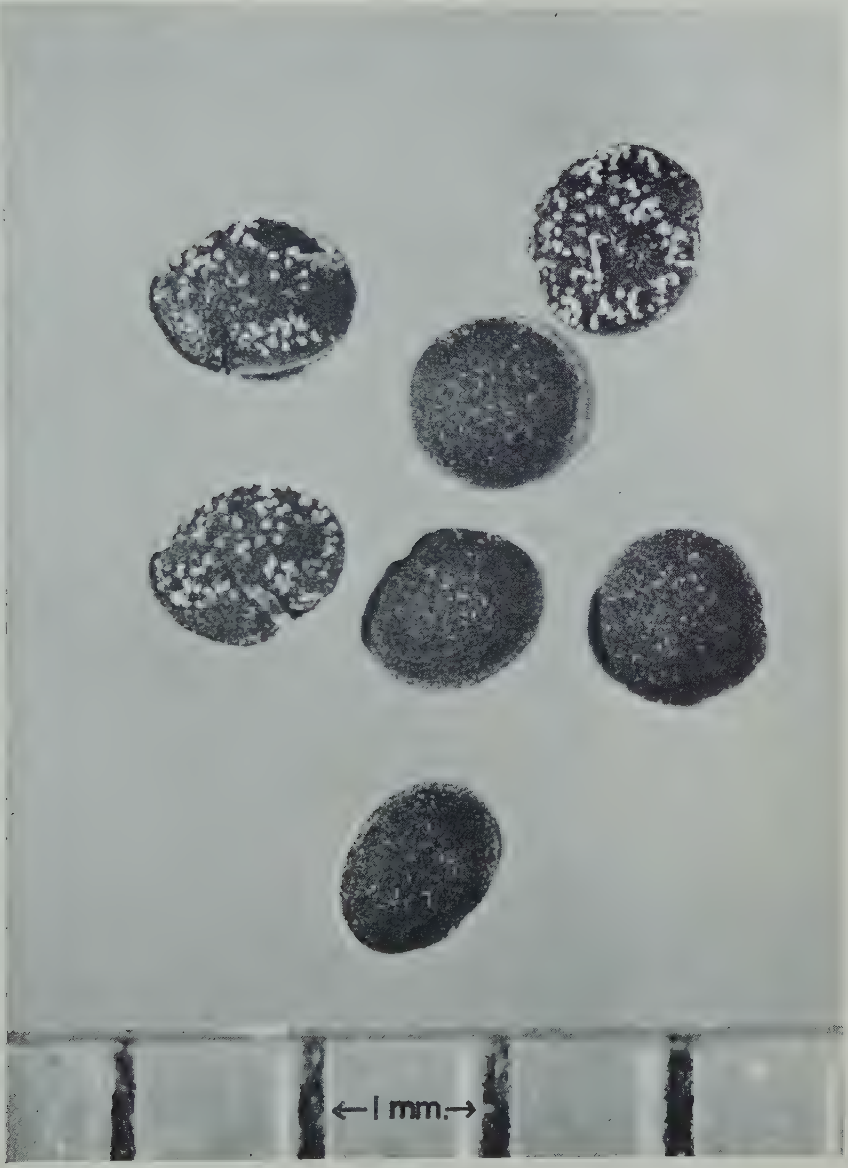

J. NEW

Three papillate and four non-papillate seeds of *Spergula arvensis*

The Mobility of Manganese in the Wheat Plant

I. Redistribution and Foliar Application

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With one Figure in the Text

ABSTRACT

The distribution of manganese in wheat plants grown in solution culture has been studied. Manganese supply from the medium was so arranged that early leaves received sufficient for normal function but subsequent growth was severely restricted by deficiency of the element. Under these conditions little or no redistribution within the plant was observed, so that the early leaves retained most or all of their manganese, and remained healthy. When manganese was applied externally to the second leaves of deficient plants a small but significant amount was translocated to the growing points. The effect was slightly enhanced by repeated rewetting of the treated leaves, but the amount moved was still small in relation to that applied and to the requirement of the plants. It appears that manganese is not readily transported by the phloem under these conditions, but this does not wholly account for the ability of some leaves to retain critical levels when a state of deficiency exists at the growing points. Possible mechanisms for this effect are discussed.

INTRODUCTION

THE widespread use of foliar sprays to correct manganese deficiency in the field suggests that the leaves of a number of plants are capable of absorbing and translocating this nutrient (Boynton, 1954) as also do studies with radio-active manganese (Mn^{54}) reported by Romney and Toth (1954).

On the other hand, manganese accumulated by leaves in the normal way appears to be unavailable to the rest of the plant, which may be severely deficient. Haas (1932) found that citrus leaves, produced when adequate manganese was available, retained up to 17 parts per million after the supply was curtailed, although young leaves, containing less than 3·3 p.p.m., were obviously deficient. Similarly Ruck and Gregory (1955) showed that manganese accumulated to the extent of 200 p.p.m. in a rooted potato leaf was unavailable to the developing axillary shoot. This is supported by the work of Bolle-Jones (1955) who likewise found no evidence for movement of manganese from the laminae of potato.

In oats, Gerretsen (1937) claimed that redistribution of manganese does not take place and Williams and Moore (1952) found no loss of this element

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[Annals of Botany, N.S. Vol. 22, No. 88 Oct. 1958.]

from leaves of this plant during senescence. That peas also behave in this way was shown by Heintze (1946), manganese accumulated by the haulm being unable to prevent the development of 'marsh spot' in the seed, which required a continuous external supply.

The only results at variance with the above appear to be those of Fudge (1939) and Smith *et al.* (1952) who found a slight fall in the manganese content of old citrus leaves during the development of fruit or new leaves, and those of Ballard (1940) who stated that dead leaves of sugar-cane contained very much less than did green tissue. In the latter case it seems possible that leaching by rain or dew could have taken place, and in the former redistribution was, at best, extremely slow.

Thus the evidence which is available on the mobility of this element is in some degree conflicting. The experiments to be described were carried out in the hope that by following simultaneously the distribution of manganese within the plant, and of that applied to the leaf surface, a clearer understanding of the problem might be gained.

MATERIALS AND METHODS

Culture

An early maturing, spring type wheat (variety Festival), supplied by the Department of Agriculture, New South Wales, was used as the test plant.

Seedlings were raised in damp sand for seven days before being suspended over 600 ml. 'Pyrex' glass beakers containing nutrient solution of the following composition.

Calcium nitrate	0·005	Molar	
Potassium nitrate	0·005	,,	
Magnesium sulphate	0·002	,,	
Disodium Phosphate	0·001	,,	
Iron	3	parts per million as	ferrous sulphate.
Boron	0·27	,, ,,	boric acid.
Zinc	0·06	,, ,,	zinc sulphate.
Copper	0·06	,, ,,	copper sulphate.
Chlorine	3·0	,, ,,	sodium chloride.
Manganese	0–2	,, ,,	manganese sulphate.
pH	5·5–6·0.		

Major nutrients were purified by the method of Stout and Arnon (1939), and water by passage through a 'Permutit' ion exchange unit. The manganese content of the purified solution was found to be 0·0015 parts per million. The medium was not aerated.

Continuous overhead light was provided by three 80-watt fluorescent tubes, supplemented by natural daylight from three sides. Temperatures varied about a mean nightly minimum of approximately 50° F., and a daily maximum of 70° F.

Transpiration losses were made up at regular intervals, but solutions were renewed only when a change in nutrient level was intended. Ferrous sulphate, equivalent to 3 p.p.m. in solution, was added each week.

Under these conditions vigorous growth was made, and the plants given adequate manganese eventually produced three or four well formed ears.

Analytical Procedure

Individual plant parts were dried for 24 hours at 80° C. for dry weight determinations, and then ashed at 500–520° C. in nickel boats prior to the estimation of manganese. This was done by a colorimetric method involving the oxidation of tetramethyldiaminodiphenylmethane (Single, 1957) using a procedure modified from that of Ruck and Bolas (1954) as follows.

Ash samples were dissolved in 1N. nitric acid and an aliquot of this solution not exceeding 0·25 ml. and containing between 0·025 and 0·1 μg. of manganese was made up to 10 ml. with sodium acetate-acetic acid buffer (pH 4·8, 0·75 M. with respect to sodium acetate). This was maintained at a temperature of 25±0·5° C. To this mixture was added 0·5 ml. of saturated potassium periodate solution, and 0·1 ml. of 1 per cent. solution of tetrabase in acetone. The intensity of the blue colour developed in 20 to 40 seconds was measured at its maximum in an E.E.L. colorimeter, calibrated at frequent intervals against a known standard of manganese sulphate.

Experimental

Uniform seedlings were grown for 15 days in a medium containing approximately 0·006 p.p.m. manganese. The solution was then changed, reducing the available manganese to 0.0015 p.p.m. Eight days later symptoms of manganese deficiency, in the form of greyish streaks of water soaked tissue, appeared in the leaves of several plants. At this stage stem elongation had not begun, and all plants had 4 leaves, with the fifth visible in a few cases. Nine plants were selected at random and divided into tillers, roots, leaves one to four, and remainder (i.e. undeveloped leaves five and six, stem, and ear). This material was dried and stored for analysis at the completion of the experiment, and is designated 'initial sample' in the presentation of results.

The remaining plants were submitted to the following treatments, each with nine replicates.

Treatment 1 (*Control*): Plants were transferred to fresh nutrient solution. This was intended to be of the same initial composition as that in which they had previously been growing, but was found later to contain 0·004 p.p.m. of manganese. The source of this contamination was not traced, but as the same solution was used for the other treatments it did not seriously affect the experiment.

Treatment 2: The second leaf of each plant was painted on each surface with a 0·5 per cent. solution of $MnSO_4 \cdot 3H_2O$, using a soft brush and making sure that the surface was thoroughly wetted. The culture beakers were covered with two layers of aluminium foil, and the base of each plant wrapped first in cotton wool and then with a strip of foil to prevent contamination of the solution.

Treatment 3: This was identical with the above, but the treated leaf was sprayed on 12 consecutive days with pure water. This was done to ensure that penetration of the cuticle was not restricted through lack of moisture. It was found that the leaves, after the initial wetting by the manganese solution, were readily rewetted by the spray, which spread evenly over the surface.

Treatment 4: Manganese sulphate was added to the solution to raise the manganese concentration to 0·25 p.p.m.

Harvest

After a period of 20 days there were obvious differences in growth between the various treatments. Plants in Treatment 4 were green and healthy while severe symptoms of manganese deficiency, in the form of necrotic streaks and chlorosis, were obvious in the remainder. A visual estimate of the proportion of necrotic tissue in each of the leaves on the primary shoot was made, and plants were divided into tillers, roots, leaves one to six, stem, and ear, for estimations of dry weight and manganese content.

TABLE 1

Mean Dry Weights of Plant Parts (mg.)

		Treatments			
		1	2	3	4
	Initial sample	Control no Mn	2nd leaf painted	2nd leaf painted and rewet	0·25 p.p.m. Mn in soln.
Tillers	32·2±6·91	261·4±26·5	249·6±24·7	237·6±51·4	679·1±62·8
Roots	100·0±6·33	111·6±6·37	125·5±5·27	118·5±10·67	250·4±13·56
Leaf 1	13·0±0·37	15·3±0·69	15·5±0·73	13·4±0·56	15·6±0·80
Leaf 2 (blade)	13·7±0·34	14·2±0·64	16·0±0·68	15·0±0·94	—
Leaf 3	37·1±3·55	38·7±2·74 (7)	42·7±3·00	38·6±2·00	43·4±2·05
Leaf 4	67·3±2·55 (8)	73·3±2·37 (7)	77·6±4·13 (7)	76·1±2·41	86·9±2·26 (8)
Leaf 5	—	90·6±2·24 (7)	98·7±2·92 (7)	105·0±2·30 (7)	144·4±4·12 (8)
Leaf 6	—	79·4±6·12 (7)	89·9±5.92 (7)	105·8±3·40 (7)	172·4±7·40 (8)
Stem	34·7+leaf 5, 6 and ear	95·4±7·17 (5)	83·6±4·63 (7)	96·1±9·57 (7)	189·3±13·5 (8)
Ear	—	7·1±1·77 (5)	5·79±0·42 (7)	7·93±1·75 (7)	29·3±2·56 (8)
Total	299·9	789·0	808·8	815·3	1,625·8

Nine replicates in each sample except where shown otherwise by bracketed figure.

Results

Results are shown in Tables 1–4 in terms of dry weight, manganese content as micrograms and as parts per million of dry weight, and estimated severity of symptoms.

This experiment was in many respects a repetition of an earlier one which lacked Treatment 3 (i.e. no spraying with water was included) and in which

TABLE 2

Mean Manganese Contents of Plant Parts (μg.)

		Treatments		
	Initial sample	1 Control no Mn	2 2nd leaf painted	3 2nd leaf painted and rewet
Tillers	0·18	0·55±0·060	0·68±0·070	0·75±0·105 (7)
Roots	1·44±0·08	1·52±0·09	1·61±0·08	1·90±0·312 (8)
Leaf 1	0·185±0·025	0·212±0·026	—	—
Leaf 2 (blade)	0·353±0·019	0·265±0·019	—	—
Leaf 3	0·460±0·038	0·474±0·030 (7)	0·460±0·022 (7)	0·496±0·028 (7)
Leaf 4	0·37±0·052 (8)	0·392±0·016 (7)	0·413±0·025 (7)	0·449±0·008 (7)
Leaf 5	—	0·264±0·013 (7)	0·309±0·016 (7)	0·357±0·008 (7)
Leaf 6	—	0·164±0·016 (7)	0·216±0·020 (7)	0·258±0·015 (7)
Stem	0·241±0·012 +leaf 5, 6 and ear	0·141±0·007 (5)	0·158±0·011 (7)	0·166±0·009 (7)
Ear	—	0·021±0·003 (5)	0·025±0·005 (7)	0·031±0·006 (7)
Total	3·23	4·01	4·36	4·59

Nine replicates in each sample except where shown otherwise by bracketed figure.

stems and ears were bulked for analysis. These results are omitted for reasons of space, and because they showed an almost identical pattern of manganese distribution to that reported here.

No manganese estimations were made on plants from Treatment 4. It may be assumed from previous results, in which values of 30 p.p.m. for the sixth leaf, and 70 p.p.m. for the second were recorded, that a manganese content in the solution of 0·25 p.p.m. is adequate under the conditions prevailing.

GROWTH IN RELATION TO MANGANESE CONTENT

From Table 1 it is clear that growth was severely limited by withholding manganese from the solution, and that this could not be made good by applying this element to the second leaf although as much as 50 micrograms were added in this way, equivalent to between 2,000 and 3,000 p.p.m. dry weight.

Tables 3 and 4 show the relations between manganese content and the development of symptoms. Although growth of leaves took place with as little as 2 p.p.m., five times this amount was necessary for continued health of the tissue. It was noted that once a leaf had been produced at suboptimal levels, i.e. below 10 p.p.m., the later addition of manganese to the solution did not prevent necrosis.

TABLE 3

Mean Manganese Contents of Plant Parts (p.p.m. Dry Weight)

		Treatments		
	Initial sample	1 Control no Mn	2 2nd leaf painted	3 2nd leaf painted and rewet
Tillers	4·91	2·09±0·073	2·71±0·127	3·12±0·36 (7)
Roots	14·8±0·84	14·0±1·08	13·8±0·57	14·3±0·61 (7)
Leaf 1	14·6±2·28	13·7±1·56	—	—
Leaf 2 (blade)	25·8±1·35	18·6±1·22	—	—
Leaf 3	12·68±0·74	12·34±0·67 (7)	10·94±0·61 (7)	12·9±0·78 (7)
Leaf 4	5·47±0·23 (8)	5·35±0·17 (7)	5·31±0·27 (7)	5·93±0·20 (7)
Leaf 5	—	2·91±0·12 (7)	3·12±0·098 (7)	3·41±0·073 (7)
Leaf 6	—	2·08±0·093 (7)	2·39±0·093 (7)	2·52±0·066 (7)
Stem	7·36±0·54 +leaf 5, 6 and ear	1·50±0·078 (5)	1·90±0·096 (7)	1·79±0·129 (7)
Ear	—	3·84±0·63 (5)	4·14±0·59 (7)	4·26±0·86 (7)

Nine replicates in each sample except where shown otherwise by bracketed figure.

TABLE 4

Visual Estimates of Manganese Deficiency Symptoms shown by Individual Leaves, as Mean Percentage of Necrotic Tissue

	Treatments			
	1 Control, no Mn	2 Leaf 2 painted	3 Leaf 2 painted and rewet	4 0·25 p.p.m. Mn in soln.
Leaf 1	0	0	0	0
Leaf 2	0	0	0	0
Leaf 3	trace	trace	trace	trace
Leaf 4	55	60	50	75
Leaf 5	55	65	50	0
Leaf 6	50	50	40	0

THE REDISTRIBUTION OF MANGANESE WITHIN THE PLANT

Comparison of Treatment 1 with the initial sample shows the redistribution of manganese within the plant over a period of 20 days.

Little use was made of manganese accumulated in the early leaves, although there appears to have been a significant fall in the content of the blade of Leaf 2. This conclusion is uncertain owing to the presence of silica which may have interfered with the estimation procedure, and which was found in

Leaves 1 and 2 in considerable amounts; so much so that ash from the sheathes of the second leaves fused in the furnace and could not be recovered. A further complication was introduced by variation in the size of the first leaves, those in Treatment 1 being significantly larger than their counterparts in the initial sample, purely as a result of chance. This is reflected in the dry weights and manganese contents.

It is probable that Table 3 presents the best picture of what has taken place: in both leaves concentrations have been maintained above the critical level, and they have remained healthy in spite of very severe shortage in the rest of the plant. Some re-export may have occurred, but this, at best, was very slight.

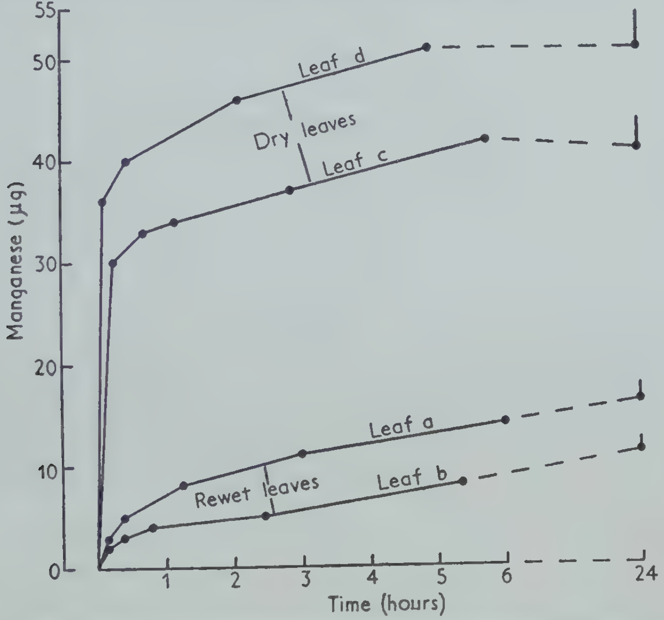

FIG. 1. Manganese dissolved from painted and rewet leaves immersed in water for 24 hours.

The amount of manganese remaining in each leaf is shown by the length of the vertical line above the final point for manganese in solution. Scale units are the same for both cases.

During the experimental period approximately 0·7 μg. manganese was taken up from the solution, of which 0·35 μg. went to the primary shoot apex, largely by-passing the fully expanded leaves. There was a slight tendency to accumulate manganese in the ears, which showed considerable variation, ranging from less than 2 to 5 p.p.m.

THE UPTAKE OF MANGANESE APPLIED TO THE LEAF SURFACE

Only a small proportion of the manganese applied to the second leaf was translocated to the rest of the plant, and uptake appeared to have been improved by rewetting the surface. An analysis of variance, based on the manganese contents of Leaves 3 to 6 in Treatments 1, 2, and 3 (Table 2) showed treatment effects to be highly significant, but the interaction treatment *v.* leaves was extremely small, indicating that no preferential distribution as between early and later formed leaves had taken place.

A second analysis, based upon the manganese contents of the severely deficient leaves only (i.e. nos 4 to 6) showed that the differences between Treatments 1 and 2, and 2 and 3 almost reached the 5 per cent. level of significance, suggesting that uptake may have been improved by rewetting.

To test this, two painted leaves from each treatment, after drying, were placed in 50 ml. each of water and stirred at intervals. From time to time the water was sampled for manganese content. After 24 hours the leaf residues were washed, ashed, and their manganese contents determined.

The results of these determinations are shown graphically in Fig. 1. It is seen that the concentration of manganese in the water rises steeply during the first few minutes in both sets of leaves, but the curves then show a sharp inflexion and within 15 minutes display a much slower rate of increase which continues for some hours. In the rewet leaves, however, the initial rise is but a fraction of that in Treatment 2, although the subsequent increase is at a comparable rate.

Clearly there has been a large net loss of manganese from the rewet leaves. This can only have come about by the water spray, actuated by compressed air, blowing droplets of manganese solution from the surface, as run-off was not observed; translocation to the rest of the plant (approximately 0·3 μg.) may be ignored.

The effect of this treatment was thus the reverse of that expected, as after 24 hours' extraction the leaves which were not rewet appeared to contain considerably more manganese.

It seems reasonable to assume that the steady rise in manganese content of the extract, after the initial rapid increase, was due to diffusion out of the leaf following on the dissolution of that deposited on the surface. If this is so, then by extrapolating back to zero time an estimate of total external manganese is obtained. Subtracting these figures from the appropriate totals it is then possible to derive estimates of the amount of soluble manganese within the leaves. These are:

Treatment 4 (rewet).	Leaf *a*	12·5 μg.	or	694	p.p.m.	D.W.
	Leaf *b*	8·7 μg.	,,	544	,,	,,
Treatment 3 (not rewet).	Leaf *c*	11·7 μg.	,,	731	,,	,,
	Leaf *d*	11·7 μg.	,,	616	,,	,,

These calculations suggest that considerable quantities of manganese had entered the leaves, but again fail to show any increased penetration as a result of rewetting. Thus another explanation for the apparent superiority of the rewet plants must be sought. A possibility is that as only the second leaf was rewet, and the plants were transpiring vigorously at the time, some water was withdrawn from this leaf and carried manganese with it in the xylem.

DISCUSSION

While confirming the general lack of mobility claimed for manganese by other workers, these results establish differential rates of movement from similar leaves, dependent upon the conditions of supply.

When manganese was applied in excess to the outside of the second leaf, a significant quantity found its way to the growing points. It is reasonable to suppose that the bulk of this transport was effected by the phloem, as there was no suggestion of preferential distribution to Leaves 3 and 4, which were the only ones capable of effective transpiration at this time.

The amount moved from the painted leaf was still very small in relation to the concentration of soluble manganese within the tissue, and it can only be assumed that phloem transport had reached saturation at a rate in the vicinity of 0·5 micrograms of manganese in 20 days. This is obviously very minute in comparison with the normal rates of transport of sugars, or of the mobile inorganic nutrients such as potassium, nitrogen, or phosphorus, and places manganese in the same category as calcium, which is moved with great difficulty, if at all, in the phloem (Arisz, 1952).

However, a very much lower rate, possibly down to zero, was observed in the transport of manganese from leaves containing only minimal amounts, and this cannot have been due entirely to restrictions imposed by the phloem. It may well be that an element which enters into so many phases of metabolism (Hewitt, 1951) tends to accumulate at reaction centres remote from the phloem elements, when present only in critical concentrations. Some evidence in support of this comes from the work of Hagen *et al.* (1952) who showed that isolated wheat chloroplasts are capable of concentrating manganese from solution. Such a mechanism would have the effect of further reducing the concentration gradient along the phloem, which may itself be a factor in reducing rate of movement from leaves which are not richly supplied.

Some further evidence bearing upon this point will be reported in a later contribution.

ACKNOWLEDGEMENTS

Thanks are due to Professor F. G. Gregory for suggesting the problem and providing many constructive ideas, to Dr. B. D. Bolas and members of the Research Institute of Plant Physiology, and to Dr. F. R. Tubbs, Director, and members of East Malling Research Station, where the work was carried out.

The project was made possible by the award of the Farrer Memorial Scholarship for Agricultural Research.

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The Mobility of Manganese in the Wheat Plant

II. Redistribution in Relation to Concentration and Chemical State

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With one Figure in the Text

ABSTRACT

Wheat plants were grown in solution culture at two levels of manganese supply until the second leaves were fully expanded. The supply of manganese was then curtailed and symptoms of deficiency appeared in subsequent growth. Although redistribution of manganese at rates dependent upon initial content occurred in both high- and low-manganese plants, first and second leaves always retained sufficient for normal function, and remained healthy. In a second experiment no redistribution from the second leaves was observed under similar conditions of supply and demand.

When second leaves were killed and placed in water, up to 80 per cent. of the manganese diffused out, but extraction of similar leaves with 80 per cent. methanol removed only 15–20 per cent. of that present. These results suggest that the lack of mobility shown by the element is not due to precipitation or complex formation within the tissue, although at high levels of manganese supply the solubility of manganese silicate might be exceeded, and some precipitation may occur.

INTRODUCTION

IN Part I of this study (Single, 1958) it was shown that fully expanded wheat leaves, which contained little more than the minimum quantity of manganese required for healthy growth, i.e. 10–15 p.p.m. dry weight, retained most or all of this in spite of severe deficiency at the growing points. However, when excess of the element was applied externally to similar leaves, a significant amount of translocation was effected. Thus a quantitative difference in rate of export was established between leaves which had been supplied in different ways. From the data available it was not possible to determine whether this was due to differences in the manner of uptake of manganese, i.e. whether from the roots or via the leaf surface, or whether it was simply a function of the concentrations within the leaves, as those supplied externally contained very much greater amounts.

The experiments to be described were designed to study redistribution from comparable leaves which had accumulated either minimal or excess amounts of manganese via the roots, and to provide, at the same time, information regarding the chemical state of the element within the tissue.

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[Annals of Botany, N.S. Vol. 22, No. 88, Oct. 1958.]

EXPERIMENTAL

In the first experiment (Exper. 1) wheat seedlings were raised in sand as in previous experiments, but were so arranged in the culture vessels that approximately half the root system of each plant was in manganese-free solution, while the remainder grew in nutrient containing either 2·0 or 0·05 p.p.m. manganese. They were allowed to grow until sample analyses showed that more than 60 p.p.m. manganese were present in the second leaves of the 'high' manganese plants (2·0 p.p.m. in solution), whereupon the roots dipping in the manganese solution were severed. Three days later the first sample was taken (Harvest 1). This sample was treated in 3 ways:

The first group of 20 plants, 10 each from the high- and low-manganese cultures, was dried for estimation of total manganese in the various parts, using the same technique as before, except that 50 per cent. HCl was used in place of 1N. HNO_3 for dissolving ash samples. This change was made when it was found that higher oxides of manganese could be formed on ashing material containing excess of this element.

From the second group, consisting of 6 plants of each type, the second leaves were removed and placed, in pairs, in sealed tubes. They were then frozen at −20° C. for 2 hours, transferred to 50 ml. of water, chilled rapidly, and held at 1°–2° C. for 24 hours. After this period the leaves were removed from the water, washed, dried at 80° C., weighed and ashed for manganese estimation.

The extracts were concentrated by evaporation, transferred to weighed 5 ml. 'Pyrex' beakers and when almost dry a known amount (between 45 and 55 milligrams) of 'Specpure' calcium carbonate was added. They were then dried at 80° C. to determine the weight of material which had diffused out. It was found that approximately 40 per cent. of the total occurred in this fraction.

After weighing, the extracts were ashed at approximately 450° C., dissolved in acid, and manganese contents determined. The calcium carbonate was added to prevent absorption of manganese by the glass, which can occur readily at ashing temperatures if this precaution is not taken. This technique was used throughout in determining manganese contents of liquid samples. It was found to give reliable results as long as allowance was made for the presence of excess calcium ions, which can cause interference in the estimation procedure used.

The second leaves from a third group, consisting of 4 high manganese plants only, were weighed fresh and then ground in an agate mortar with 80 per cent. methanol. The homogenate so formed was transferred to a 10 mm. × 50 mm. Soxhlet thimble and extracted under reduced pressure with 80 per cent. methanol for 24 hours.

Manganese contents of extract and residue were then determined. The liquid fraction appeared to contain all the chlorophyll and other pigments. In preliminary experiments on similar material the methanol was removed from the extract by boiling under reduced pressure, with repeated addition of water. This brought about the precipitation of the chlorophyll, along with other water-insoluble compounds. Over 90 per cent. of the manganese, however, remained in the aqueous phase, regardless of the initial concentration in the tissue.

In presenting the data from this experiment the figures have been corrected for traces of manganese present in the thimbles, and in the methanol which was redistilled before use. Contamination from these sources was very slight.

The sequence of operations which went to make up this experiment was as follows.

Day 0. (26–3–'57). 200 grains were sown in damp sand.

Day 7. 120 seedlings were transferred to solution culture as described, 70 in high manganese (i.e. part of root system in 2·0 p.p.m. Mn solution) and 50 in low-manganese solution (0·05 p.p.m.). A further 9 seedlings were dried for initial estimation of manganese in the roots.

Day 18. Roots dipping in solutions containing manganese were severed. At this stage the third leaf of the majority of plants was half grown.

Day 21. 20 high- and 16 low-manganese plants were sampled for analysis as described (Harvest 1). The remainder were placed in fresh culture solution, without manganese, with the exception of 10 plants from the high-manganese group which were given 0·25 p.p.m. manganese (control).

Day 25. Symptoms were observed in 2nd, 3rd, and 4th leaves of the majority of the plants in the low-manganese treatment. Only occasional high-manganese plants showed damage to 3rd and/or 4th leaves.

Day 30. The manganese control was clearly superior in growth and colour to the high-manganese treatment, which was superior to the low. Symptoms were visible in the 5th leaf of all low-manganese treatment plants, and in a few of the high manganese group. All 5th leaves in the control were healthy.

Day 35. 20 high- and 16 low-manganese plants were harvested and treated as before (Harvest 2).

Day 77. 10 high-manganese, 10 low-manganese, and 10 control plants were harvested and dried (Harvest 3).

In spite of the long period between the first and last harvests, nutrient solutions were not changed; however, ferrous sulphate was added weekly, and other trace elements, including molybdenum, were given at fortnightly intervals.

At the completion of the experiment control plants had produced three or four well formed ears each, and grain was filling normally. Most of the first and second leaves had undergone senescence, but others were dark green and healthy.

In the 'high' manganese treatment (2·0 p.p.m. in solution before transfer), plants were chlorotic with large areas of necrotic tissue. Ears were small, usually only one per plant, and showing no ability to fill grain. Senescence had occurred in some first and second leaves, but to a markedly less extent than in the controls.

Many plants of the 'low' manganese treatment (0·05 p.p.m. in solution before transfer) were dead, except for the first and second leaves, which were mostly still green as senescence was even less evident than in the high-manganese treatment. In the majority of cases roots were brownish and flaccid, and ears had not emerged from the sheath.

TOTAL MANGANESE CONTENT OF PLANT PARTS

In Table 1 the results of the first part of this experiment, concerning the total manganese content of the various plant parts, are presented. Several estimates are marked with an asterisk and this shows that they are to some extent unreliable due to difficulties experienced with manganese estimations in the presence of silica.

In practically all estimations undertaken on plants from the later harvests, significant quantities of silica were present in the ash. To overcome this, each ash sample was first extracted with 50 per cent. HCl, and the manganese content determined. The silica was then centrifuged off, washed, and fused with sodium carbonate in a platinum spoon or crucible. This converted silica to sodium silicate, setting free manganese for estimation in the normal way.

As a rule the silica fraction accounted for less than 5 per cent. of the total manganese, and is included in the figures given. In three cases, however, inaccuracy was introduced due to contamination of the platinum crucible, so that estimates can only be based on the first acid extract of the ash. These are the ones marked with asterisks, and may be regarded as valid minima. The origin of the silica was not determined, but as the water was purified only by a single passage through a Permutit Portable 'Deminrolit' Plant some contribution from this source was to be expected.

From the results in Table 1 a number of features of manganese distribution can be deduced. These may best be discussed individually in relation to the organs concerned.

Distribution of manganese from the roots

The figures shown in Table 1 refer only to the roots which were growing in manganese-free solution after the others had been removed. Some additional data are available, however, concerning the status of the roots before this stage had been reached.

At the time the seedlings were transferred to solution culture, 9 plants were sampled and those parts of their root systems which corresponded to those placed in manganese-free beakers were analysed. The results were as shown in Table 2.

As the manganese contents of the roots were determined on four separate dates it is of interest to examine these data graphically as in Fig. 1.

From these curves it is seen that while all the root systems were intact (i.e. up to Day 18), those in the manganese-free beakers of the high-manganese

Table I

Mean Dry Weights and Manganese Contents of Plant Parts at Three Harvest Dates

	Low manganese			High manganese			Control
	Harvest 1	Harvest 2	Harvest 3	Harvest 1	Harvest 2	Harvest 3	Harvest 3
Roots							
Dry wt. mg.	33·8±2·10	64·4±4·60	114·3±13·42	41·2±2·47	95·9±6·02	140·2±11·06	413·9±35·1
Mn µg.	0·374±0·019	0·637±0·032	1·458±0·150	0·689±0·107	0·940±0·111	1·744±0·109	—
Mn p.p.m.	11·33±0·79	10·04±0·28	13·30±1·43	16·72±2·48	9·36±0·70	12·82±0·87	—
Leaf (1)							
Dry wt. mg.	22·0±1·28	19·6±0·69	25·9±1·21	21·2±0·53	20·3±0·75	18·9±1·92	16·7±1·53
Mn µg.	0·677±0·058	0·596±0·045	0·487±0·044	2·15±0·124	1·89*±0·287	1·15*±0·176	—
Mn p.p.m.	30·7±1·40	30·6±2·49	18·8±1·45	101·8±5·93	90·9*±12·16	62·5*±13·70	—
Leaf (2)							
Dry wt. mg.	29·3±1·35	31·5±1·23	39·6±2·47	31·6±0·85	32·6±1·45	35·9±2·41	26·5±2·07
Mn µg.	0·612±0·083	0·571±0·033	0·527*±0·034	1·90±0·142	1·40±0·136	1·08±0·104	—
Mn p.p.m.	21·1±3·07	18·6±0·83	13·3*±0·42	60·4±4·74	44·8±4·14	30·54±2·65	—
Shoots							
Dry wt. mg.	63·1±5·55	319·8±18·2	795·6±71·1	73·5±3·33	467·2±21·0	1,111·2±71·54	4,466±259
Mn µg.	0·914±0·062	1·654±0·065	3·014±0·226	2·426±0·376	2·866±0·188	3·835±0·204	—
Mn p.p.m.	14·8±0·72	5·23±0·168	3·85±0·215	33·0±5·20	6·14±0·274	3·48±0·088	—
Total							
Dry wt. mg.	148·2±7·90	435·3±23·32	975·4±85·85	167·5±6·58	616·0±26·66	1,307·2±88·91	4,923·2±291
Mn µg.	2·58±0·154	3·46±0·118	5·72±0·357	7·17±0·626	7·09±0·634	7·93±0·418	—

Ten replicates in each sample except for harvest 3, high manganese, where nine only were available.
* = minimum estimate from first acid extract.

treatment took up appreciably more of this nutrient than did the corresponding roots of the low; i.e. approximately 0·3 μg. per plant.

TABLE 2

Analysis of Roots in Manganese-free Solution at Day 7

Mean dry weight	4·3±0·36 mg.
Mean manganese content	0·215±0·032 μg.
Mean manganese concentration . . .	48·7±3·86 p.p.m.

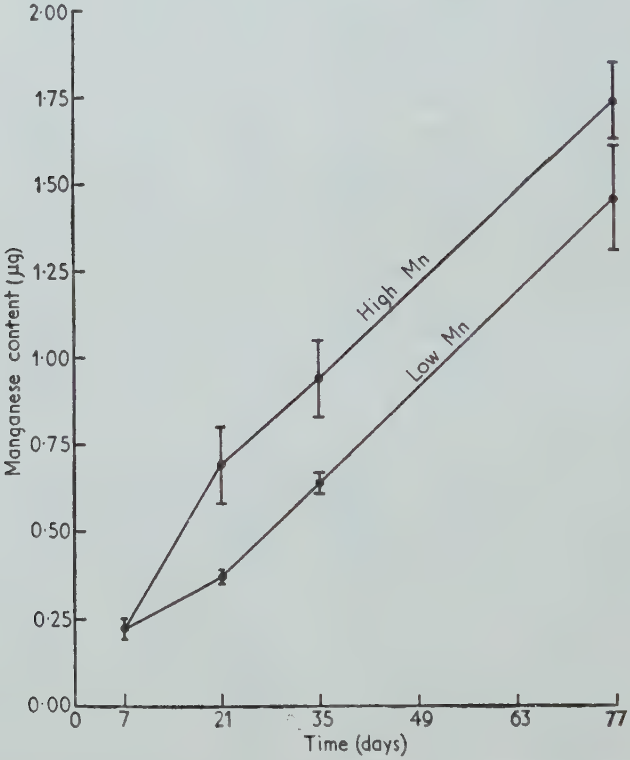

FIG. 1. Mean manganese content of roots growing in manganese-free solution.

After cutting, however, both types of roots gained manganese, presumably from the traces left in the solution, at the same rate. The fact that this rate for the low-manganese plants was actually higher than before cutting suggests that the low-manganese roots might have obtained all their manganese in this way, receiving no benefit from their counterparts growing in 0·05 p.p.m. Mn solution.

If this is true, only that amount of manganese which represents the difference between the high- and low-manganese plants can be regarded as having been transferred from one set of roots to another, i.e. 0·689–0·374 or 0·315 μg. This indicates that manganese did not move readily in the phloem of the roots, or else that there was little tendency to accumulate it away from the point of absorption, as analyses showed that over 10 μg. was accumulated in those roots which were in 2·0 p.p.m. Mn solution, and an additional 4 μg. was translocated to the rest of the plant.

For the rest, the behaviour of the roots followed a pattern that might have been anticipated from the earlier experiments. High initial concentrations were quickly reduced, either by further growth or translocation, and steady values of the order of 10 p.p.m. were maintained in the face of much lower concentrations, 5 p.p.m. or less, in the remainder of the plant. Although the roots of some plants appeared quite dead, there was no suggestion that these contained less manganese than those which were still alive, so apparently no manganese was set free by the dying tissue.

Movement of manganese from the first leaf

Although values for manganese content of the first leaf show a downward trend with time in both high- and low-manganese treatments the significance of this effect is difficult to evaluate.

In the low-manganese plants errors were small and the figures can be accepted with a degree of confidence. The analysis of variance showed that the effect of time on manganese content exceeded the 5 per cent. level of significance, so a small export of the element had probably occurred. The large drop in manganese concentration may be explained partly on this basis and partly as a result of increased dry weight. The reason for this may be associated with reduced translocation of carbohydrate and ash constituents due to the death of the rest of the plant.

Little definite information can be gained from the analyses of the first leaves of the high group due to possible losses in the silica fraction as mentioned earlier. It seems very unlikely, however, that the large apparent drop in manganese content, i.e. almost 50 per cent., can be explained entirely on this basis.

Apart from this, it is obvious that substantial quantities of manganese were retained by the leaf despite very severe shortage in the rest of the plant. This is shown by the mean figure for manganese concentration, which, even at the end of the experiment, was four times that required for healthy growth. Of interest also is the very large error associated with these estimations. In fact the concentrations at final harvest ranged from 31·2 to 163·0 p.p.m., and appeared to be in no way associated with the degree of senescence of the individual leaves.

Movement of manganese from the second leaf

The manganese content of the second leaf in the high-manganese group fell throughout the experiment in a manner similar to that shown by the first. In this case, however, there is no reason to doubt the accuracy of the estimations, and the effect is highly significant statistically. Again, the final concentration was well in excess of critical levels, and this was maintained in the face of a demand within the plant which existed, as shown by deficiency symptoms, for more than seven weeks.

In the low-manganese plants the second leaf did not lose a significant proportion of its manganese, although, if the complication due to silica is ignored, there is a suggestion of a slight fall with time. As in the first leaf a rise in the dry weight towards the end of the experiment accentuated the apparent decrease in concentration. Perhaps the only worthwhile conclusion which can be drawn from the behaviour of this leaf is that manganese contents, which were little above critical levels at the commencement of the experiment, were largely maintained.

Redistribution of manganese within the plant

The section of Table 1 designated 'shoots' embraces all remaining parts of the plant, which were bulked for analysis. It is to be expected that if the fall in manganese content of the first and second leaves was real, then a corresponding rise should have occurred in this fraction.

This was seen to be the case, so that total manganese content of the high plants showed little change throughout the experiment, growth depending upon internal redistribution of manganese present at the beginning. These figures, however, have little significance due to considerable variation between plants. This is shown by the large standard errors appropriate to the different means. It appears that plants vary greatly in their uptake of manganese under conditions of more than adequate supply.

In the low-manganese plants a different situation was observed as a significant increase, of approximately 3 μg. of manganese, occurred during the experimental period. This is somewhat larger than was to be expected from the original manganese content of the solution, or of the water used to replenish transpiration losses, and suggests contamination, perhaps from dust, as the beakers were not sealed. In view of the large errors associated with parallel figures for the high-manganese plants, it is not possible to determine whether the apparent difference in uptake of manganese, as shown by the two treatments, is a real one.

2. SOLUBILITY OF MANGANESE IN LEAF TISSUE AS DETERMINED BY EXTRACTION

Tables 3 and 4 show the amounts of manganese remaining in and diffusing out of second leaves after the cells had been killed by freezing. Samples were taken at the first and second harvest dates, from both high- and low-manganese plants.

Although these plants were sown and harvested at the same time as those described in the first part of this experiment, they were not completely comparable with them. As the principal aim of this work was to establish the amount of redistribution, if any, which occurred, the best and most uniform plants were selected for dissection and direct analysis. Those used for the freezing and diffusion tests were slightly smaller at the time of setting up, and served as buffer plants at each end of the bench. This may account in some part for the generally lower total manganese contents observed, although considerable variation was to be expected from the behaviour of the supposedly more uniform material.

Table 3

Diffusion of Manganese into Water from Second Leaf after Freezing, High-manganese Plants

	Dry wt. mg./leaf	Mn. μg./leaf			Mn. p.p.m. dry wt.			% Extracted
		Residue	Extract	Total	Residue	Extract	Total	
Harvest 1	24·5	0·47	1·035	1·505	19·2	42·2	61·4	68·8
	33	0·64	1·625	2·265	19·4	49·2	68·6	71·7
	31·5	0·375	0·815	1·190	11·9	25·9	37·8	68·5
Mean	29·7	0·495	1·16	1·65	16·8	39·1	55·9	69·7
Harvest 2	27	0·380	0·380	0·76	14·0	14·0	28·1	50·0
	27·5	0·45	0·555	1·005	16·4	20·1	36·5	55·2
	27·5	0·52	0·84	1·36	18·9	30·6	49·5	61·8
Mean	27·3	0·450	0·59	1·04	16·4	21·6	38·0	55·7

Table 4

Diffusion of Manganese into Water from Second Leaf after Freezing, Low-manganese Plants

	Dry wt. mg./leaf	Mn. μg./leaf			Mn. p.p.m. dry wt.			% Extracted
		Residue	Extract	Total	Residue	Extract	Total	
Harvest 1	26	0·14	0·185	0·325	5·4	7·1	12·5	56·9
	37·5	0·25	0·30	0·55	6·7	8·0	14·7	55·6
	31·5	0·19	0·175	0·365	6·0	5·6	11·6	47·9
Mean	31·7	0·193	0·22	0·41	6·0	6·9	12·9	53·5
Harvest 2	30·5	0·20	0·17	0·37	6·6	5·5	12·1	45·9
	32·5	0·26	0·215	0·475	8·0	6·6	14·6	45·3
	31·5	0·20	0·19	0·39	6·3	6·1	12·4	48·7
Mean	31·3	0·22	0·19	0·41	7·0	6·0	13·0	46·6

Table 5

Manganese Extracted from Leaves by Reflux with 80 per cent. Methanol

	Dry wt. mg./leaf	Mn. μg./leaf			Mn. p.p.m. dry wt.			% Extracted
		Residue	Extract	Total	Residue	Extract	Total	
Harvest 1	26·5	1·88	0·345	2·225	70·94	13·02	83·96	15·51
Harvest 2	29·7	1·283	0·378	1·660	43·11	12·69	55·80	22·74

For this reason little significance can be attached to the apparent fall in total manganese content which occurred between the first and second harvests in the high-manganese group (Table 3). By the same token the indication that the whole of this fall was borne by the soluble fraction must be treated with some reserve. It is clear, however, that considerable quantities of manganese, in a readily soluble form, were present in the leaves during the course of the experiment.

The low-manganese plants (Table 4) showed a somewhat similar pattern, except that there was no suggestion of a fall in total manganese content between the two harvest dates. Again this is in general agreement with the behaviour of the main part of the experiment. Here, too, the soluble manganese made up a very considerable fraction of the total. This would appear to be of some importance, because in this case the total concentration was the same as has been shown to be critical in the health of the leaf.

The effect of aqueous methanol extraction of leaf tissue is shown in Table 5. Of principal interest is the small amount of manganese extractable by this technique. When this was brought to light by the analysis of the first set of leaves it seemed possible, although unlikely, that the extraction had not gone to completion in the 24 hours. The leaves from the second harvest were therefore extracted for a further 12 hours with fresh 80 per cent. methanol. No appreciable quantity of manganese could be detected in the second extract, so it may be concluded that the figures shown represent the whole of that fraction which was soluble in aqueous methanol. As preliminary experiments had shown that manganese was not precipitated from a similar extract on removal of the methanol, it may be concluded that this fraction was also soluble in water and was probably in the form of free ions.

Thus results from the two extraction procedures contrast strongly. When simple diffusion into water was employed more than 50 per cent. of the manganese was removed from the leaf, and this fraction appeared to be diminished with time. When methanol was employed, however, only approximately 20 per cent. of the manganese was soluble and this showed a proportional increase as the overall content of the leaf was depleted by redistribution within the plant.

EXTRACTIONS BY WATER AND METHANOL

In order to examine further the apparent difference in the effects of water and 80 per cent. methanol in extracting manganese from leaves, 48 plants were raised, using the same technique as applied in Experiment 1 up to the stage of the first harvest, i.e. 3 days after severing of roots. Although every effort was made to keep conditions similar to those which applied previously, this was not possible due to unusually hot weather during the initial stages. As a result the second leaves were very much smaller than expected, but nevertheless took up manganese to the extent of 200 p.p.m., or more than twice the concentration observed in the earlier experiment.

At Harvest 1 the second leaf blades of 24 plants were removed in 6 groups each of 4 leaves. They were split longitudinally, weighed, and the 4 half-leaves from each group sealed in a separate tube. Thus 6 pairs of tubes were prepared, the tubes of each pair containing virtually identical material.

Immediately upon sealing, the tubes were placed in an ice-salt bath at −30° C. for 2 hours. After freezing, the leaves from one tube of each pair were transferred to 50 ml. of water (plus a trace of Teepol to act as a wetting agent) and from the other to 50 ml. of 80 per cent. methanol. They were then chilled rapidly and stored at 0°–2° C. for 24 hours. At the end of this period the leaves were removed, washed, and placed in a fresh 50 ml. of water or methanol as before.

TABLE 6

Diffusion of Manganese from Leaves on Two Occasions into Water and 80 per cent. Methanol

Figures, in μg. per leaf, are the means of 6 determinations, each on 4 half leaves

	Diffusion into water				
	Residual	1st extract	2nd extract	Total extract	Total Mn
Harvest 1 . .	0·53	1·89	0·14	2·03	2·56
Harvest 2 . .	0·65	2·05	0·11	2·16	2·81

	Diffusion into 80% methanol followed by water					
	Residual	1st MeOH	2nd MeOH	Water	Total Extract	Total Mn
Harvest 1 .	0·59	0·22	0·04	1·84	2·10	2·69
Harvest 2 .	0·63	0·49	0·09	1·56	2·14	2·77

After a further 24 hours the leaves in the water were washed, dried, weighed, and ashed for determination of residual manganese. Those in methanol were transferred to 50 ml. of water and stored at 0°–2° C. for 48 hours before being dried and ashed for manganese determination.

The remaining 24 plants were grown for a further period of 14 days, during which time symptoms of deficiency appeared in the later-formed leaves. The second leaves were then treated as above.

RESULTS

The results of this experiment in terms of mean manganese contents per leaf of the various fractions are shown in Table 6. Data from the two harvests are generally in close agreement and appear to show a slight overall rise in manganese content during the experimental period, the reverse of what was expected. By combining the total manganese contents for water and methanol treatments the error appropriate to this comparison can be calculated. This is:

Mean manganese content (μg.)	Harvest 1	2·625
	Harvest 2	2·788
	Difference	0·163
Standard error of the difference between means		0·0829
Difference for significance	$P = 0·05$,	0·185
	$P = 0·10$,	0·150

From this it appears that the difference may well be ascribed to chance, and the fact that some manganese was lost in the analysis of the residue samples at Harvest 1, makes this even more likely. The only significant difference which is shown between the two harvest dates is in the methanol treatment, the amount of methanol-soluble manganese having more than doubled with time at the expense of the water-soluble fraction.

The fact that the second methanol extracts were in each case less than 20 per cent. of the first suggests that manganese was present in the leaf in three different states, confirming the results of the previous experiment. The first of these was insoluble in water or aqueous methanol and no further evidence as to its chemical state is available. The second was soluble in both solvents and was probably largely in the form of free manganese ions, accompanied by the more soluble anions such as chloride. The third was insoluble in 80 per cent. methanol, but soluble in water, and made up a substantial portion of the total manganese present.

TABLE 7

Amounts of some Elements Present in Extract in Relation to Amounts Present in a Whole Sample of Apple Leaves

Elements present in relatively high concentration	Moderate amounts present	Trace only present
Si, P, Mo, Ca	Mg, Mn, Al, Sn, Cu, Ag, Zn	K, Na, Fe, V

The best source of this third fraction was obviously the water extract following methanol. This solution, which was clear and colourless and contained 0·062 p.p.m. manganese, was examined as follows.

1. A manganese determination was carried out in the usual way on 1 ml. of the solution, but without prior ashing. This gave a figure of 0·05 p.p.m., showing that at least 80 per cent. of the manganese present was free to enter the oxidation-reduction cycle in the estimation reaction.

2. It was concentrated 10 times by evaporation, and added to four volumes of pure methanol.

A flocculent, brownish precipitate was produced, which contained over 50 per cent. of the manganese originally present in the solution. This was readily dissolved in a little water, giving a clear solution which contained over 1 p.p.m. manganese. This solution was tested with excess of trichloracetic acid for the presence of protein. No precipitate was formed.

3. The concentrated solution was tested with ammonium molybdate, and gave a yellow precipitate, indicating the presence of phosphate.

4. Solution equivalent to 3 leaves was evaporated to dryness and examined spectrographically. Although it is not possible to determine from this single, preliminary analysis, the amounts of the various elements present in absolute terms, the spectrum could be compared with that from a normal sample of apple leaves, of similar initial dry weight, included in the same run. This allowed the comparison in Table 7 to be made.

DISCUSSION

The presence of silicon, presumably in the form of silicates, and of phosphates, suggests a likely explanation for the effects observed. Manganese silicate is probably the least soluble simple salt of manganese, a saturated aqueous solution containing only 10 to 20 p.p.m. manganese. The addition of 4 volumes of methanol reduces this figure, expressed on a water basis, to approximately 0·5 p.p.m. Although this is still above that found in the 80 per cent. methanol extract it is understandable that movement from the leaf into this solvent would be extremely slow, as the solution within the tissue would rapidly become saturated, and the concentration gradient favouring diffusion would still be relatively small.

The above results therefore suggest that a large proportion of what may be termed excess manganese in the leaf may be precipitated out as the silicate, or, more likely, as complex salts with other heavy metal silicates, phosphates and, perhaps, molybdates. The solubility of these is such that they are not extracted in the presence of excess methanol, but dissolve quite readily in water.

The marked difference in behaviour between supposedly similar plants in the two experiments shows that the mobility of manganese, even in relation to a single species, is not capable of definition in simple terms, but may depend upon processes within the plant which are unrelated to the supply of, or demand for, the element at the time. This appears to be true also for iron (Biddulph, 1951) and conflicting reports on the behaviour of copper and zinc (reviewed by Williams, 1955) suggests that it may be the case with these metals also.

The readiness with which manganese diffuses out of killed leaves, even when present only in minimal quantities, indicates that lack of mobility is not due to precipitation within the tissue, and this is in agreement with results presented in Part I of this investigation (Single, 1958). It is still possible that, at critical levels (i.e. 10–15 p.p.m.), the element may be accumulated within the chloroplasts and mitochondria, but further information is required on this point.

When the manganese content of tissue is high, i.e. more than 100 p.p.m., it appears very likely that solid manganese silicate may be deposited. The solubility of this salt is such that this reaction might have the effect of controlling manganese levels within the tissues without interfering with utilization or redistribution. Some such mechanism is suggested by the work of Williams and Vlamis (1956) who found that silicate added to the nutrient solution in which barley plants were growing favoured even distribution of manganese in the leaf and reduced toxicity effects.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Professor F. G. Gregory and Dr. B. D. Bolas for many helpful ideas, and to Dr. F. R. Tubbs and members of East Malling Research Station, where the work was carried out. Facilities for reflux extraction of tissues were made available by Dr. A. E. Flood in the Biochemistry Section, and spectrographic data were provided by Messrs. A. C. Mason and H. J. Philcox.

These studies were carried out while one of us (W. V. S.) was holder of the Farrer Memorial Scholarship for Agricultural Research.

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Variations in the Germination-rate and Development of Fern Spores in Culture

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With one Plate and one Figure in the Text

ABSTRACT

In fern prothalli growing in pure culture, a number of variations have been observed in the rapidity of the germination of spores and the subsequent growth of the prothalli. Although these variations have not all been satisfactorily explained, experiments show that it is possible to produce considerable changes in the germination and growth of fern spores by introducing fungal contamination and prothallial extracts into the media on which they are sown. These changes are considered in relation to variations in the developmental morphology of spores in pure culture and it is suggested that indole-3-acetic acid may be the active substance involved.

ALTHOUGH a number of workers have studied the development of the gametophytes of European ferns (e.g. Orth, 1936), attention has been paid almost entirely to that leading directly to the cordate prothallus. It is, of course, well known that other types of prothalli occur in some species, particularly those which fail, or which are slow to develop apices and which persist for a long time in the male condition. In the present experiments the observations have been confined to variations in the rate of development of the cordate prothalli not associated with any conspicuous male phase.

MATERIALS AND METHODS

The basic substrate used was Moore's medium (Moore, 1903), with the addition of trace elements and solidified with 2 per cent. Difco agar. The pH of the cold solidified medium lay in the range 4·5–5·5. A sterile suspension of fern spores was prepared by a standard procedure in which they were treated momentarily with 15 per cent. solution of calcium hypochlorite and washed with sterile tap water. The sowing on agar slopes was done by means of either sterile camel-hair brush or a fine pipette.

THE EFFECTS OF FUNGAL CONTAMINATION

In a series of six cultures of *Thelypteris palustris* it was observed that in one the spores had germinated well in advance of the others and the gametophytes were growing conspicuously more rapidly. At the same time a fungus, the hyphae of which formed minute tufts about 0·2 mm. in diameter, appeared on the surface of the agar in one part of the culture. The other 5 cultures of the sowing were aseptic, and germinated and grew uniformly.

[Annals of Botany, N.S. Vol. 22, No. 88, Oct. 1958.]

The fungus appearing in the sixth tube was obtained in pure culture on Knop's agar supplemented by 2 per cent. glucose. It readily formed a white weft of hyphae on the surface and, after 2 weeks, a pale brown coloration within the medium. No sex organs, fructifications, or conidia could be found. The mycelium was septate. From the uniform appearance of the fungus, both under the microscope and in successive sub-cultures, it was assumed that only one species was present. After isolation of the fungus a series of experiments were carried out to test whether fern spores sown on a medium previously inoculated with it germinated and grew faster than on sterile controls.

Experiment 1: Thelypteris palustris Schott. Small cubes (about 5 mm. long at each edge) cut from the agar just beneath the surface of a culture of the fungus were placed in a central position on three slopes of Moore's agar. Cubes of similar size of exactly the same medium as that on which the fungus was growing, but sterile, were placed on three identical slopes to act as controls. Three days later all six slopes were sown with spores of *Thelypteris palustris* and placed on a culture apparatus providing continuous illumination at an intensity of approximately 115 foot-candles.

Within a week it was clear that the spores on the inoculated slopes were germinating and growing considerably faster than on the uninoculated. At 3 weeks, when two of the slopes were extracted and photographed, the difference between the two series was considerable (Plate). By this time the fungus had made only a little growth around the fringes of the implanted cube and had not invaded the surface of the slope.

Experiment 2: Polystichum lobatum (Huds.) Woynar. Exactly the same procedure was followed as with *Thelypteris palustris*. The result was even more striking. At 3 weeks the spores on the uninoculated slopes, although still appearing normal, had not even germinated, whereas those on the inoculated had grown into cordate prothalli-bearing antheridia.

Experiment 3: Dryopteris filix-mas (L.) Schott. The results were similar to those obtained in Expt. 2, except that a cluster of spores germinated at the base of one of the control slopes. This was so conspicuous that it was thought that a chance infection of a fungus similar to that which had been isolated earlier might have occurred, but none was observed. It could, however, have been easily overlooked had it been confined to the cluster of prothalli.

It was not possible to perform any further experiments with this fungus for some time, and after the delay it was discovered that the cultures had been accidentally lost.

THE EFFECTS OF PROTHALLIAL EXTRACTS, PROTHALLIAL DEBRIS, AND OTHER SUPPLEMENTS ADDED TO THE MEDIUM

No fungus having the properties of that described in the foregoing has yet appeared again in cultures of spores. Meanwhile, experiments have been performed to investigate the growth-regulating substances associated with the prothalli themselves. A culture of *Dryopteris filix-mas*, a little over 2 years old, was available for these experiments. The spores, sown thickly on 100 ml. of medium sloped in a 500-ml. conical flask, had developed into a dense felt of prothalli about 1 cm. thick, brown and necrotic below, but still actively growing above. There was no indication of contamination with fungi or bacteria. A few of the prothalli were forming weak sporelings apogamously. The agar, prevented from drying out by capping the flask with polythene, was suffused with a brown pigment, especially adjacent to the prothalli. The felt of prothalli was removed from the agar, immediately weighed (43 gm.), and ground to a paste in a glass mortar with about a teaspoonful of thoroughly washed sharp sand. When smooth, 25 ml. of Pyrex-distilled water were slowly added and the grinding continued for a few minutes. The mixture was allowed to stand only long enough for the sand to settle; as soon as this had occurred the turbid supernatant liquid was decanted. This process was repeated with two further lots of 25 ml. of distilled water. The three extracts were pooled and finally centrifuged at 3,000 revolutions per minute for 5 minutes, yielding an almost clear, pale green, supernatant liquid and a compact brown sediment. The former was separated from the latter without disturbance, and both the water extract and the debris were incorporated into media.

Four series, each of three slopes consisting of 100 ml. of medium in all, were made up as follows:

Series 1. The control, consisting of Moore's agar as used in the experiments already described.

Series 2. The supernatant fluid obtained above (75 ml. in volume) was incorporated in 100 ml. Moore's agar.

Series 3. The sediment from the centrifuge tube was also incorporated in 100 ml. Moore's agar. The medium was agitated thoroughly when pouring the slopes so that the finely divided prothallial debris was uniformly distributed between the tubes. As the agar cooled, the debris flocculated irregularly.

Series 4. The agar on which the prothalli had been growing (which, owing to evaporation and uptake of water by the prothalli, had shrunk to about two-thirds of its original volume) was comminuted and incorporated in 100 ml. Moore's agar.

About 2 hours elapsed between removing the felted prothalli and the placing of the various media into the autoclave for sterilization.

The following day all twelve slopes were sown with spores of *Dryopteris borreri* Newm. and placed upon the culture apparatus.

RESULTS

In 1 month the spores on the medium containing the water extract of the prothalli (series 2) were clearly germinating more readily than those in any other series; no germination at all could be seen on the slopes of the series 3 or 4. At 12 weeks the prothalli of series 2 were cordate and bearing antheridia freely, whereas those of the control were smaller and had not yet entered the antheridial phase. Those of series 4 were retarded and only just becoming cordate. No germination at all could be detected in series 3.

At six months, when the experiment was discontinued, the prothalli of series 2 were producing sporelings apogamously in the normal way for this species, whereas those of the control, although now in the antheridial stage, had shown very little more growth and were beginning to appear unhealthy. The prothalli in series 4 had continued to make only very slow growth, and in series 3 the spores were still lying ungerminated on the surface of the agar.

The behaviour of the slopes in each series was perfectly consistent, with the sole exception that on one of the three control slopes there was no germination. It was almost certain that this had been accidentally passed over in sowing, since no spores could be seen on the surface of the agar at the end of the experiment.

The effect of other supplements. In an attempt to discover the nature of the growth-regulating substances detected in the foregoing, experiments were set up in which casamino acids, bactopeptone, the vitamin supplement of White's medium, glucose, and sucrose were added to the basic medium. Although a number of these substances influenced the growth-rate of prothalli, none produced effects as striking as those obtained in the earlier experiments.

Discussion

Although it has been known for some time that an extract of the fungus *Boletus edulis* promotes the germination of the spores of the moss *Funaria hygrometrica* (Heitz, 1942) and that the presence of *Penicillium* sp. hastens the appearance of the nature form of the protonema of the same species (Sironval, 1947), a general stimulation of development in the gametophyte of ferns by a fungus or fungal extract does not appear to have been described hitherto. Wilkie (personal communication) has discovered a crested variety of *Pteridium aquilinum* in which the continued development of the gametophyte occurs only when the culture is contaminated with any of a wide range of micro-organisms, but the situation revealed here differs, since the spores of the species used will grow perfectly satisfactorily in sterile conditions on simple mineral agar and only one of the many contaminants has been observed to produce the striking acceleration described.

Since normal development of these species is possible on aseptic media, it follows that the control prothalli either possessed, or were capable of synthesizing, the intermediary metabolites and enzymes essential for growth and development. Nevertheless, the presence of the fungus stimulated growth and, since the stimulation was of general growth-rate and not of some isolated feature, such as cell enlargement, an exudate from the fungus appears to have entered concordantly into the metabolism of the prothalli. There is thus a strong possibility that the exudate did not provide a metabolite or metabolites wholly wanting in the control prothalli, but supplemented a supply already available to them, but insufficient to allow maximum growth-rate in the physical conditions of the experiment. Nevertheless, the inoculation was so small, and the growth of the fungus after transference so slow, that its exudate must have been effective in very low concentration.

The results obtained with the prothallial extract, which resemble but are not so striking as those of fungal contamination, are different from those reported earlier by Döpp (1949) and Näf (1956). These workers have demonstrated that a water extract of actively growing prothalli of certain ferns is capable of causing the precocious formation of antheridia in prothalli of the same or other species, but in the experiment reported here the effect of the extract was clearly to increase the growth-rate. The difference may follow from the fact that *Dryopteris borreri* is apogamous.

The interpretation of the growth promotion. Now that it is established that prothalli grown on agar impart substances to the medium, probably an inevitable consequence of the close contact between the medium and the prothallus in its early phase, much of the curious behaviour of prothalli in culture becomes explicable. For example, it has been found that small prothalli of *Thelypteris palustris* (2 to 3 mm. in length) growing normally from a spore culture fail to grow when transplanted to fresh medium, if placed flat upon it. If this is done, the prothallus either becomes decolourized within a few days, and dies, or produces filamentous or nodular processes at its margin. On the other hand, the growth of a prothallus placed with only its posterior region in contact with the surface is not interrupted. This difference in behaviour might follow from too great a loss of essential metabolites from the prothallus in intimate contact with the medium to allow continued and ordered growth. Growth promotion should perhaps not be looked upon only as the direct supplementation of the metabolism of the gametophyte, but also as preventing, by providing a concentration in the medium, the loss of essential metabolites from the prothalli by diffusion into the medium.

Irregular development of spores is often found when the number of spores in relation to the volume of the medium is small. In *Thelypteris palustris*, for example, germination in some sowings is very poor, although the spores are of one gathering and their treatment unchanged. A possible explanation of this variation is advanced below, but the point here is that the spores of these anomalous cultures usually develop into nodular masses of cells, instead of almost immediately into cordate prothalli as normally. Cordate laminae arise from these nodules much later, but irregularly, and the appearance of such a culture is very uneven. Fig. 1 A, B show prothalli of the same age from a culture showing poor germination; Fig. 1 C a much younger prothallus from a uniform culture of the same spores developing normally. Although this striking difference may follow from the same factors which cause the sparse germination, the smallness of the number of germinating spores may itself affect their development. This view is supported by observations made on *Dryopteris filix-mas*. In an attempt to obtain undisturbed individual prothalli of this fern, single spores were sown on a series of slopes of Knop's agar, each 20 ml. in volume, while simultaneously other similar slopes were sown with the usual dense suspension. Of the four single spores which germinated, not one developed normally, but each grew slowly into a nodular mass of cells reaching about 3 mm. in diameter, eventually producing a cluster of cordate prothallial lobes over its surface, and nodular and filamentous outgrowths from its base.

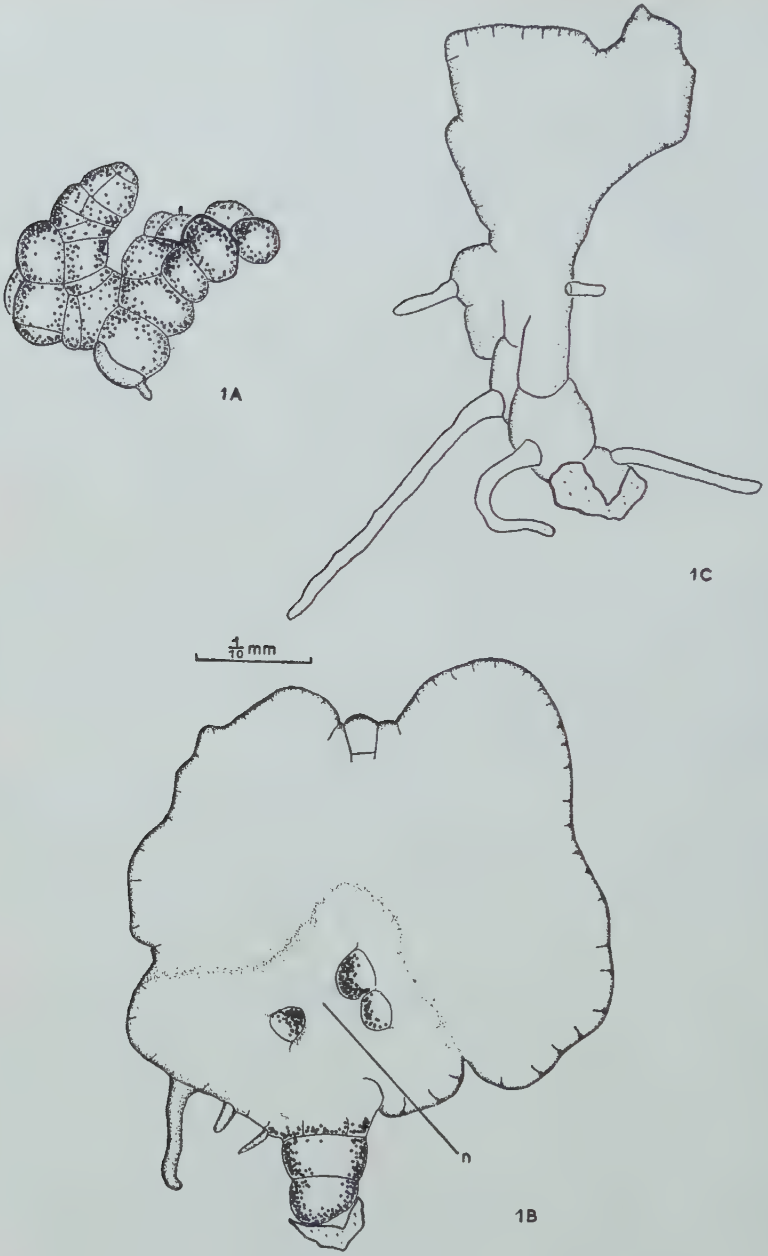

Fig. 1. *Thelypteris palustris.* Prothalli developing on a mineral agar surface. A and B from a culture showing sparse and irregular germination, each 10 weeks old, but at different stages of development; C from a normal uniform culture of the same gathering of spores for comparison, 5 weeks old. In B a cordate wing is rising from the basal nodule of cells, *n*, some of the cells of which are seen projecting above the surface. In C there is no initial nodule and the rapid development of the cordate prothallus is accompanied by the production of conspicuous rhizoids. All asexual.

These structures, never observed in bulk sowings of *Dryopteris filix-mas*, resemble the *colonies prothalliennes* of Hurel-Py (1950) and Steeves *et al.* (1955). The identity of the behaviour of the four single spores, strongly contrasting with the rapid normal development of spores of the same gathering sown in bulk at the same time, militates against its being a chance occurrence. That their slow and irregular development resulted from a prolonged loss of some metabolite into the medium appears a plausible explanation. It is noteworthy that Hurel-Py (1950), who studied a wide range of ferns, also remarks that the germination of isolated spores is considerably slower than that of grouped, and that the former are a frequent source of *colonies prothalliennes*. Moreover, she finds that the *colonies* are more readily obtained by the transplanting of small prothalli than large, as would be expected on the argument advanced above, since the concentration of diffusing substances would not fall to such a low level in a large mass of tissue as in a smaller before equilibrium was reached. In higher plants, continued ordered differentiation in culture depends upon the size of the explant (see, for example, Robbins *et al.* (1936)), and Went (1954) reports that differentiation in the protocorms of orchids in culture takes place most readily when the opportunity for diffusion from the protocorm into the medium is least. The more regular behaviour of spores on soil than on agar may also result in part from the physical unevenness of the surface of the former and the smaller opportunity for outward diffusion.

The interpretation of the growth inhibition. The behaviour of the cultures in which the medium contained either prothallial debris or agar on which prothalli had been previously grown may be explained by the production of inhibitors in prothalli. If so, the growth-inhibiting substance or substances appear to be less readily extractable with water than the growth-promoting.

The results of the extraction experiment can also be interpreted in terms of a single substance (or group of substances) different in its effects at different concentrations. If the active substance continued to be liberated from the prothallial debris as it lay in the medium, then it might have reached a high and inhibitory concentration in this series. The original agar, being greater in volume than the prothalli and therefore, if there was an approach to equilibrium between the agar and the prothalli, able to contain a greater quantity of diffusing substances, might also have supplied the medium containing it with an inhibitory concentration of the active substance.

It is noteworthy that Döpp found that the water extract of the prothalli of *Pteridium aquilinum* in high concentration inhibited growth of other prothalli. He was unable to decide whether this was an effect of the antheridia-inducing factor or of other substances.

The chemical nature of the active substance. The experiments with supplements of known nature gave no suggestion that the supply of any of the commoner amino-acids, B-vitamins, or sugars was limiting growth of the prothalli on Moore's agar.

There is a possibility that the growth promotion and inhibition observed in these experiments was brought about by indole-3-acetic acid (IAA). This auxin was first known from the products of fermentation (Salkowski, 1880) and there is strong evidence that it is normally present in fern prothalli (Albaum, 1938). Also, IAA in concentrations of 0·01 and 0·10 μg. per ml. is known to stimulate the growth of prothalli; the results of Hurel-Py (1943), for example, indicate a doubling of the growth-rate of those of *Asplenium* sp. when grown on Knop's agar supplemented with 0·01 μg. per ml. of IAA. Also IAA in concentrations above 1·0 μg. per ml. interferes with the germination of spores (Sossountzov, 1957) and diminishes the rate of growth of prothalli (Hurel-Py, 1943).

It is a common experience of those raising fern spores in pure culture that the extent of their germination and the regularity of their subsequent growth varies from one sowing to another. The causes of this variation are obscure, but since most workers have maintained their cultures in more or less constant conditions, and used for their media salts of standard analytical purity, it is assumed (as, for example, recently by Mohr (1956)) that the explanation lies in variations in the quality of the agar. Agar is known to yield growth-promoting substances in the presence of certain proteolytic enzymes (Kulescha, 1948). It is possible that substances of this kind liberated in varying amounts from different samples of agar as a consequence of enzymes diffusing from germinating spores are another cause of their erratic behaviour in culture.

The varying proportions of aberrant prothalli obtained when different species are sown on the same medium may follow from differences in metabolism between them. At the same time purely physical factors may play a part. The behaviour of the spores of *Thelypteris palustris* and *Dryopteris filix-mas* in agar cultures is conspicuously more regular than that of the spores of *Pteridium aquilinum*; the spores of the former possess a perispore, those of the latter do not. This proteinaceous layer may prove an effective impediment to the diffusion of metabolites from the spore as it lies on the agar surface.

The author is indebted to Miss E. Davies and Miss J. Bilby for technical assistance and to his colleague Dr. D. Wilkie for making his observations upon *Pteridium aquilinum* freely available for discussion.

SUMMARY

1. Controlled inoculation of mineral media with a fungus obtained from a chance contamination on an agar slope showed that in its presence the spores of *Thelypteris palustris*, *Polystichum lobatum*, and *Dryopteris filix-mas* germinated and grew faster than in aseptic conditions.

2. Supplementing the mineral medium with the commoner amino acids and the vitamin supplements used in tissue culture did not produce an effect similar to that of the fungus.

3. A water extract of old prothalli of *Dryopteris filix-mas* promoted the growth of *Dryopteris borreri*, but the prothallial debris and the agar on which the spores had been growing strongly inhibited germination and growth.

4. The interpretation of the mechanism of growth promotion and develop-

ment in general in the conditions of growth on an absorptive agar surface is discussed.

5. It is suggested that in these experiments indole-3-acetic acid may have been causing both the promotion and inhibition of growth, depending upon the concentration in which it was present.

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EXPLANATION OF PLATE

Thelypteris palustris. An inoculated (*left*) and control slope (*right*), photographed 24 days after sowing the spores.

P. R. BELL

The Carbohydrate Nutrition of Tomato Roots

V. The Promotion and Inhibition of Excised Root Growth by Various Sugars and Sugar Alcohols

BY

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With one Figure in the Text

ABSTRACT

Iron is only consistently present in an available form in White's root culture medium if the inorganic salts are autoclaved with the sugar. The substitution of ferric ethylenediamine-tetra-acetate for the inorganic ferric salt of White's medium ensures iron availability when the carbon source of the medium is rendered sterile by ether treatment and subsequently added to the remainder of the constituents which have been sterilized by autoclaving.

The biological activity of sugars, and particularly of dextrose and laevulose, is altered by autoclaving them in presence of the inorganic salt solution of White's medium.

The only sugar which supports a considerable growth of excised tomato roots is sucrose. The activity of this sugar is not affected by alcohol-precipitation nor is it dependent upon the simultaneous presence of traces of its constituent monosaccharides.

Dextrose or laevulose or a mixture of the two sugars support a low but sustained level of excised-root growth. All other sugars and sugar alcohols tested were inactive as carbon sources.

The addition of sucrose at low concentration (0·2 per cent.) to a medium containing dextrose as the main carbon compound does not make possible a level of growth comparable with that obtained with an adequate sucrose supply. It has not been possible to enhance the growth-rate of excised roots supplied with dextrose by previous presentation of this sugar under conditions permitting active growth. Using media containing 'etherized' sucrose and dextrose, no evidence was obtained of any competitive inhibition of sucrose utilization by dextrose.

Certain sugars when added to a medium, containing the optimum concentration of sucrose, markedly inhibited excised root growth. Thus *l*-sorbose, *l*- and *d*-arabinose, and *d*-xylose caused not less than 80 per cent. inhibition at a concentration of 0·5 per cent. *d*-mannose and *d*-galactose completely inhibited growth at 0·1 per cent. The oligosaccharides, dextrose, laevulose, and the sugar alcohols tested had, by contrast, very low inhibitory activity.

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[**Annals of Botany, N.S. Vol. 22, No. 88, Oct. 1958.**]

INTRODUCTION

EARLIER papers in this series have presented evidence that sucrose is an effective source of carbon and energy for the growth of excised tomato roots and that no other sugar, tested, supports a comparable level of growth (Dormer and Street, 1949; Street and Lowe, 1950). Dextrose was found to support a very slow but sustained rate of growth. Laevulose, according to the sample tested, either completely failed to support growth or promoted a slow rate of extension growth for only a very limited period. Galactose, mannose, arabinose, xylose, rhamnose, and maltose were found to be without any growth-promoting activity as sole carbon sources (Street and Lowe, 1950). These results pointed to the high specificity of the sucrose requirement of excised tomato roots.

Street and Lowe (1950) also noted that galactose, xylose, laevulose, and maltose markedly inhibited excised root growth when supplied in the presence of sucrose. Dextrose was less inhibitory but appeared to act as a competitive inhibitor of sucrose utilization.

The range of carbohydrates involved in these earlier experiments was very incomplete and the sugars, with the exception of sucrose and dextrose, were tested at only a single concentration. All sugars, with the exception of sucrose in certain experiments, were sterilized by autoclaving the complete culture medium. Observations published by Burström (1941), Evans (1942), and Sattler and Zerban (1945, 1949), however, raise the question of how far the physiological activity of sugars may be altered by autoclaving in presence of the salts and organic supplements of root culture media. These considerations prompted the more detailed and comprehensive examination, now reported, of the effects on excised root growth of sugars and sugar alcohols.

EXPERIMENTAL

General experimental procedures

The general culture technique was as described in earlier papers (Street and Lowe, 1950; Street and McGregor, 1952). A clone of excised tomato roots derived from a single seed of the doubled haploid variety San Marzano was used throughout.

The ferric sulphate of White's medium (1943) was replaced by an equivalent amount of ferric chloride (as the spectrographically standardized solution of Johnson Matthey & Co.) or by ferric ethylenediaminetetra-acetate (FeEDTA) at a concentration giving 1 p.p.m. iron (Sheat, Fletcher, and Street, 1958).

The following methods of sterilization have been employed:

(*a*) Autoclaving of the complete medium at 15 lb./in.^{2} for 10 minutes.

(*b*) Autoclaving of the medium in excess of its final strength and with all or part of the carbohydrate addition omitted. Completion of the medium by the aseptic addition to this of a sterile aqueous solution of carbohydrate prepared by one of the following procedures:

(i) Autoclaving of the carbohydrate in aqueous solution ('*autoclaved separately*').
(ii) Sterilization of the carbohydrate in aqueous solution by filtration (Street, McGonagle, and Lowe, 1951) ('*filtered*').
(iii) Treatment of the dry carbohydrate with Analar ethyl ether, removal of the ether at a temperature below 30° C., and solution of the carbohydrate in sterile double-distilled water ('*etherized*').

Root growth was allowed to proceed for 7 days at 27° C.±0·5, except where otherwise explicitly stated. The features of growth measured were: increase in the main axis length (mm.) (I.M.A.), number of laterals per root (L.N.), and total length of laterals per root (mm.) (L.L.). In most experiments each treatment was represented by 9 replicate cultures and the growth values shown are mean values for these replicates.

Sucrose as a carbon source

Optimum growth of the present clone of excised tomato roots was obtained by maintaining the clonal 'sector' cultures in medium containing 1·5 per cent. sucrose and by using 1·0 per cent. sucrose for the experimental 'tip' cultures.

TABLE 1

The Growth of Roots in Media Containing 1·0 per cent. Sucrose

Experiment No.	Sucrose supply	Technique of sterilization	Replicate No.	Mean growth values		
				I.M.A.	L.N.	L.L.
C4	'Analar'	Autoclaving	20	209±3·4*	95·8	611
	Alcohol-precipitated	Autoclaving	18	212·1±4·3	93·9	598
C8	'Analar'	'Etherized'	19	232·4±3·5	57·2	513
	Alcohol-precipitated	'Etherized'	18	224·9±4·5	58·8	514

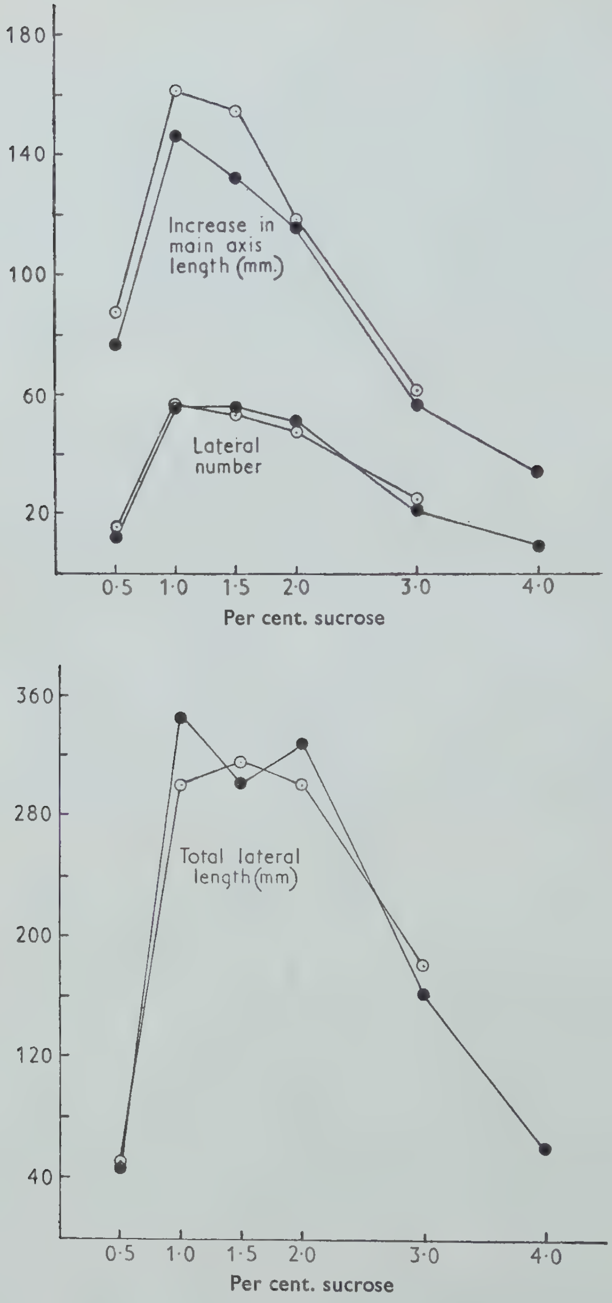
* Standard error.

The choice of 1·0 per cent. sucrose for the 'tip' culture passages was dictated by the responses to sucrose concentration shown in the figure. Dry weight yield showed an optimum at 2·0 per cent. sucrose. At 1·5 per cent. and higher concentrations there was a tendency for the roots to brown when cultured in medium sterilized by autoclaving.

Using media containing FeEDTA as iron source and sterilized by autoclaving the whole, or media prepared with 'etherized' sucrose, it was shown that 'Analar' sucrose and this sucrose purified by alcohol precipitation (Dormer and Street, 1949) were of equal growth-promoting activity (Table 1). These results indicate that the specific requirements for sucrose is not dependent upon impurities in the sucrose sample or upon its partial hydrolysis.

Iron availability in White's medium

Variable and, on occasions, very poor growth was obtained in White's medium prepared by addition of 'filtered' sucrose to the remaining constituents sterilized by autoclaving as a mixed solution at 1·25 × the final strength.



The effects of sucrose concentration on the growth of the excised roots of the tomato variety, San Marzano.
Results of two experiments shown.

'Etherized' sucrose similarly incorporated also gave, from occasion to occasion, media varying in growth-promoting activity. When 'etherized' alcohol-precipitated sucrose was used the medium consistently failed to support growth.

TABLE 2

Restoration of the Growth-promoting Activity Resulting from Addition of Monosaccharides to White's Medium containing 'etherized' Alcohol-precipitated Sucrose (1·0 per cent.)

Treatment	Replicate No.	Mean growth values		
		I.M.A.	L.N.	L.L.
Autoclaved medium	9	162·0±9·4	56·4	250
'Etherized' sucrose	9	21·4±9·5	4·7	14
,, +0·05% dextrose	9	160·4±9·2	62·0	392
,, +0·01% laevulose	10	151·1±10·1	52·8	282
Autoclaved medium	10	184·8±9·3	62·6	431
'Etherized' sucrose	10	7·9±7·0	0	0
,, +0·05% *d*-xylose	8	179·9±6·7	51·6	363
,, +0·1% *l*-rhamnose	9	120·4±1·0	57·4	294
,, +0·1% *l*-arabinose	10	125·4±8·9	44·7	234
,, +0·1% *d*-arabinose	10	79·4±4·9	44·4	224

The reducing sugar limit of 'Analar' sucrose (British Drug Houses Ltd.) is given as 0·01 per cent. calculated as $C_6H_{12}O_6$. Monosaccharide contamination would be reduced by alcohol precipitation. Experiments were, therefore, undertaken to see if the addition of monosaccharides to the fraction of the medium which is autoclaved would restore the growth-promoting activity of medium containing 'etherized' alcohol-precipitated sucrose. The following monosaccharides effected more or less complete restoration of the activity of this otherwise inert medium: laevulose, dextrose, *d*-xylose, *l*-arabinose, *d*-arabinose, and *d*-rhamnose. The fully effective concentrations of these sugars and the growth values in their presence are shown in Table 2. By contrast the following compounds were inactive: *d*-mannose, *d*-galactose, α-methyl-*d*-glucoside.

TABLE 3

The Growth-promoting Activity of 0·01 per cent. Laevulose autoclaved with the Salts and Vitamins, 'autoclaved separately' and 'etherized'

Treatment	Replicate No.	Mean growth values		
		I.M.A.	L.N.	L.L.
Autoclaved medium	9	178·2±7·3	79	632
'Etherized' sucrose	9	6·9	0	0
'Etherized'+laevulose				
(i) 'etherized'	9	6·6	0	0
(ii) 'autoclaved separately'	9	5·9	0	0
(iii) 'autoclaved'	9	176·5±10·2	83	733

The question then arose as to whether the active monosaccharides were directly effective or whether they gave rise to active substances during autoclaving in presence of the salts and vitamins of the medium. To test this, 0·01 per cent. laevulose was incorporated in the medium as previously and in the same experiment also by being 'autoclaved separately' and 'etherized'. The results shown in Table 3 indicate that the laevulose is only active when autoclaved with the other medium constituents. When laevulose was, in a subsequent experiment, autoclaved with the separate individual salts and the vitamin mixture of the medium, activation only occurred where it was autoclaved with the ferric chloride. The other active monosaccharides were then shown to behave similarly, being inactive when incorporated by being 'autoclaved separately' or 'etherized' and being active if autoclaved with either the total salt mixture or with the ferric chloride alone. Further experiments revealed that higher concentrations of laevulose and dextrose developed a low level of activity even when 'autoclaved separately'; this was in contrast to similar concentrations incorporated by 'etherizing'.

TABLE 4

The Growth-promoting Activity of Sugar Acids at a Concentration of 0·002 per cent. when 'autoclaved separately' or 'etherized'

Treatment	Replicate No.	Mean growth values		
		I.M.A.	L.N.	L.L.
'Etherized' sucrose	8	7·5	0	0
+glucono-Δ-lactone, 'etherized' .	9	118·1	39·0	302
+glucuronic acid, 'etherized' .	9	121·1	37·0	303
,, 'autoclaved separately' .	10	116·4	38·6	427
+galactono-Δ-lactone, 'etherized' .	9	95·3	30·4	242
,, 'autoclaved separately' .	9	127·5	50·6	379
+galacturonic acid, 'etherized' .	9	110·1	19·6	106
,, 'autoclaved separately' .	9	150·8	50·6	435

These results suggested that the sugars gave rise to an active compound in low yield when heated in aqueous solution and in greater yield in presence of ferric chloride. Possible derivatives under these conditions are sugar acids and tests with glucono-Δ-lactone, glucuronic acid, galactono-Δ-lactone, and galacturonic acid revealed that these were active in restoring the growth-promoting activity at 0·002 per cent. concentration (Table 4). The activity of these compounds further suggested that activation of monosaccharides on autoclaving with ferric chloride might not only be due to the iron salt acting as an oxidizing agent but that the sugar derivative might also be growth-promoting because of its chelating activity for iron.

Tests were then made as to the iron availability in media containing 'etherized' alcohol-precipitated sucrose. It was then shown that full growth-promoting activity was obtained provided FeEDTA was used as iron source or provided the ferric chloride of the standard medium was autoclaved separately from the inorganic salt mixture (Table 5). These results showed that the 'active' monosaccharides when autoclaved with the inorganic salts and vitamins prevent the precipitation of iron which otherwise occurs on autoclaving the salts in presence of ferric chloride. Unpublished results indicate that up to 0·1 per cent. of reducing sugars (expressed as $C_{16}H_{12}O_6$) can be formed in 1 per cent. sucrose medium during autoclaving. The availability of iron in White's medium as normally prepared depends upon the presence of sucrose, probably because of the formation, following partial hydrolysis, of chelating compounds.

TABLE 5

Excised Root Growth in Media Containing 'etherized' Alcohol-precipitated Sucrose and either Ferric Chloride or Ferric Ethylenediaminetetracetate (FeEDTA) added before or after Autoclaving of the Main Medium Fraction

Iron source	Main medium fraction autoclaved	Aseptic addition after autoclaving	Mean growth values I.M.A.	 L.N.	 L.L.
Ferric chloride	Complete medium	Nil	157·0	60·7	534
	Medium minus sucrose	Sucrose	7·5	0	0
	,, ,, ,,	Ferric chloride + sucrose	160·3	63·1	566
	,, ,, ,,	FeEDTA + sucrose	158·6	42·3	582
FeEDTA	,, ,, ,,	Sucrose	192·7	66·2	480

These observations emphasize not only the desirability of using FeEDTA to ensure iron availability in root culture media but also that the autoclaving of sugars, particularly in the presence of salts, should be avoided when determining their biological activity.

Examination of the ability of various carbohydrates to act as sources of carbon and energy for excised root growth

The carbohydrates were tested in medium of pH 4·5–4·8 containing FeEDTA as iron source. The carbohydrates were incorporated either 'etherized' or 'autoclaved separately'.

When the 10 mm. root tips used to initiate cultures are placed in a carbohydrate-omitted medium they increase in length by up to 5 mm. and remain white and floating. After 7 days' incubation most but not all the apices have lost the ability to resume growth when transferred to the standard medium containing sucrose. Carbohydrates are reported here as incapable of supporting growth where it has not been possible, by their use, to obtain main axis extension significantly in excess of that occurring in carbohydrate-omitted medium. If, in a particular experiment, a somewhat greater initial extension has been recorded (e.g. in a particular experiment with lactose, the average I.M.A. value was 11 mm.), the decision that the carbohydrate is inactive has been based on further tests and on the demonstration that the carbohydrate, compared with the carbohydrate-omitted medium, does not prolong retention of the ability of the root meristems to resume growth in presence of sucrose. On this basis the following substances can be classified as unable to function as carbon sources: *l*- and *d*-arabinoses, dextrin, dulcitol, *d*-galactose, inositol, lactose, maltose, *d*-mannose, mannitol, α-methyl-*d*-glucoside, raffinose, *l*-rhamnose, *l*-sorbose, sorbitol, *d*-xylose. Less extensive but clear results have also indicated the following to be inactive: fructose-1-6-diphosphate, glucose-1-phosphate, glucose-6-phosphate, cellobiose. In the presence of certain samples of maltose and raffinose main axis increases of up to 25 mm. have been recorded. This could not be repeated with other samples. With these favourable samples extension growth ceased before the end of the 7-day incubation period and with prolongation of the culture period all root meristems lost their ability to resume growth when transferred to sucrose medium. Chromatographic examination suggests that the apparently low activity of samples of these oligosaccharides is due to contamination with traces of dextrose or to limited decomposition in the culture medium. With most of these inactive carbohydrates the root tips remain white and turgid during the 7-day incubation. The tips brown in presence of galactose and loose turgor in presence of the sugar phosphates.

TABLE 6

The Growth of Roots in Media Containing Dextrose as Sole Carbon Source

Treatment	Replicate No.	Mean growth values I.M.A.	L.N.	L.L.
Control (1% sucrose)	9	169·4±5·7	70·4	263
0·25% dextrose	9	12·2±2·1	0	0
0·5% „	9	22·1±3·5	0	0
0·75% „	9	33·8±4·0	1·8	6·3
1·0% „	9	36·8±8·5	1·1	5·1
1·25% „	9	45·0±3·1	2·2	5·6
1·5% „	9	47·9±4·3	4·0	11·0

Dextrose supports a slow rate of growth giving a root of delicate structure with laterals absent or few in number (Table 6). When the culture period is extended for 28 days growth continues, particularly through the few laterals initiated on the original main axis.

Because of the sustained but slow growth-rate in dextrose an attempt was made to 'adapt' a portion of the clone to this sugar by the use of media containing both sucrose and dextrose. Each passage contained 6 'sector' cultures and 15 'tip' cultures. At the end of each passage, 10 of the tip cultures were measured and discarded. The 'sector' cultures were used to initiate 'tip' cultures and the 5 remaining 'tip' cultures to re-establish 'sectors'. The experiment was continued through 14 subcultures using, in order, media of the following carbohydrate compositions; 1 per cent. sucrose+1 per cent. dextrose (4 subcultures), 1 per cent. sucrose+2 per cent. dextrose (2 subcultures), 0·5 per cent. sucrose+2 per cent. dextrose (4 subcultures), 0·2 per cent. sucrose+2 per cent. dextrose (3 subcultures), 2 per cent. dextrose (1 subculture). With each increase in the dextrose:sucrose ratio, the average growth declined (Table 7) and there was no evidence of an 'adaptation' permitting more active growth with dextrose alone. The inability of a low concentration of sucrose to enhance markedly the growth of excised roots supplied dextrose must be borne in mind when attempting to explain the unique growth-promoting activity of sucrose as a carbon source.

TABLE 7

Growth Values Recorded during Repeated Subculture in Media Containing an Increasing Dextrose : Sucrose Ratio

Passage No.	Carbohydrate supply	Mean growth values		
		I.M.A.	L.N.	L.L.
4	1% sucrose+1% dextrose	61·9	26·5	177
6	1% sucrose+2% dextrose	44·8	20·8	132
8	0·5% sucrose+2% dextrose	30·9	9·5	47
11	0·2% sucrose+2% dextrose	19·5	5·9	23

Laevulose was, in the experiments of Street and Lowe (1950), virtually inactive as a carbon source. This was confirmed in the present study using autoclaved medium. When, however, 'etherized' laevulose was used it promoted a persistent main axis extension growth slightly superior to that obtained with dextrose (Table 8). The roots were of delicate structure and devoid of laterals. Mixtures of dextrose and laevulose were not superior as a carbon source to either sugar separately.

TABLE 8

Growth of Roots in Media Containing 'etherized' Laevulose or Dextrose as Sole Carbon Source

Treatment	Replicate No.	Mean growth values		
		I.M.A.	L.N.	L.L.
Control (1% sucrose)	8	168·4±9·4	42·3	326
Laevulose 0·5%	9	60·3±6·3	0	0
,, 1·0%	8	61·4±5·8	0	0
,, 2·0%	7	70·0±6·2	0	0
Dextrose 0·5%	9	25·0±2·6	0	0
,, 1·0%	8	55·9±6·5	4·3	10·4
,, 2·0%	8	54·3±6·0	0	0

Inhibition of excised root growth from addition of various carbohydrates to sucrose containing medium

The more detailed study of this question undertaken in the present investigation has shown that certain carbohydrates, particularly dextrose and laevulose, have a more inhibitory effect upon growth when autoclaved in the medium than when incorporated by being 'etherized' or 'autoclaved separately'. Apparently the substances formed from these sugars during autoclaving and which in low concentrations are important in chelating iron are at higher concentrations markedly inhibitory to growth.

The growth effects reported below are all from experiments in which the carbohydrate has, in each case, been incorporated in the medium by a technique which does not enhance its inhibitory action. The use of FeEDTA throughout was designed to ensure that the results are not complicated by problems of iron availability. 1·0 per cent. sucrose was present in all media.

Despite some variation, from occasion to occasion, in the quantitative responses of the roots to the carbohydrate additions, it is possible to divide the substances tested into groups according to their inhibitory activity. For most of the substances listed, more than one sample has been tested.

Substances having low inhibitory activity (Group I) include dextrose, lactose, raffinose, maltose, mannitol, dulcitol, and α-methyl-*d*-glucoside (all these at 1·0 per cent. usually caused less than 30 per cent. reduction of the growth values) and also laevulose, *l*-rhamnose, and sorbitol (at 1·0 per cent., these usually caused 40–60 per cent. reduction of the growth values). Then came a group (Group II) which caused not less than 80 per cent. reduction of growth values when added at a concentration of 0·5 per cent. and which usually were significantly inhibitory at 0·1 per cent. This group comprised *l*-sorbose, *l*- and *d*-arabinose, and *d*-xylose. Group III comprised *d*-mannose and *d*-galactose, both of which were completely inhibitory at 0·1 per cent.

The use of 'etherized' sucrose and dextrose in media containing FeEDTA clearly showed that Street and Lowe (1950) only obtained data suggestive of a competitive inhibition by dextrose of sucrose utilization as a consequence of the autoclave sterilization of media containing these two sugars.

DISCUSSION

The results presented in this paper are confirmatory of earlier studies in that they emphasize the unique activity of sucrose as a source of carbon and energy for the growth of excised tomato roots.

The elimination of autoclaving in presence of the other medium constituents as the method of sterilizing the sugar component(s) and the adoption, instead, of an 'etherizing' technique has emphasized the desirability of stabilizing iron availability in root culture media by the use of FeEDTA. It has also shown that laevulose is as effective as dextrose in supporting excised root growth and that dextrose does not, contrary to the suggestion of Street and Lowe (1950), competitively inhibit sucrose utilization. The contention of Street and Lowe (1950) that roots cultured with sucrose have a sharp pH optimum for growth at pH 4·8–5·0 is also now known to be a reflection of the relationship of pH to iron availability and not indicative of the pH sensitivity of a surface-located enzyme (Sheat, Fletcher, and Street, 1958). Thus, two of the observations considered by these workers to support the tentative hypothesis of sucrose uptake by phosphorolysis (Dormer and Street, 1949) have found alternative explanations.

The problem raised by the activity of sucrose in relation to excised root growth remains and recent work with excised roots of *Senecio vulgaris* (Skinner, 1953), *Medicago sativa* (David, 1954), and *Trifolium pratense* (Dawson,

1958) suggests that this specificity of sucrose may extend to many dicotyledonous excised roots.

The growth-promoting activity of sucrose is not reproduced by the addition of catalytic amounts of sucrose to a dextrose medium nor by the use of dextrose-laevulose mixtures (both sugars incorporated by 'etherizing' and therefore free from the toxicity developed during their autoclaving). Nor are traces of mono-saccharide necessary for the sucrose medium to display its full growth-promoting activity.

Certain sugars, particularly mannose and galactose and, to a less extent, xylose and the arabinoses are highly active as root growth inhibitors when added to a sucrose medium. By contrast, the corresponding sugar alcohols have only very low inhibitory activity. A more detailed study of the toxicity of these sugars will be reported in the next paper of this series.

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The Carbohydrate Nutrition of Tomato Roots

VI. The Inhibition of Excised Root Growth by Galactose and Mannose and its Reversal by Dextrose and Xylose

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ABSTRACT

The addition of 0·01–0·015 per cent. galactose or 0·005–0·01 per cent. mannose reduces by 50 per cent. the linear growth of excised tomato roots cultured in a 1 per cent. sucrose medium. An addition of 0·03–0·04 per cent. of either sugar causes not less than a 90 per cent. inhibition of growth. The survival of meristematic activity is higher in presence of fully inhibitory concentrations of mannose than of galactose. Roots inhibited by galactose are distinguishable from those inhibited by mannose.

The inhibitory effect of concentrations of galactose up to 0·15 per cent. and of mannose up to 0·4 per cent. can be fully antagonized by the simultaneous addition to the culture medium of dextrose. The minimum ratio of dextrose: inhibitory sugar for maximum antagonism of the growth inhibition is with galactose 5:1 and with mannose 3·5:1. Growth of roots in a dextrose-containing medium does not protect them from subsequent inhibition by either galactose or mannose.

d-xylose has significant activity as an antagonist of mannose inhibition and even more so of galactose inhibition. However, the restoration of growth achievable from the addition of xylose is not comparable with that resulting from the addition of dextrose. The inhibition of growth by xylose is not alleviated by the simultaneous addition of dextrose. Maltose has low activity as an antagonist of galactose and mannose inhibition. All other sugars tested and the sugar alcohols corresponding to galactose and mannose were quite inactive as antagonists of the growth inhibition by these two sugars.

Mixtures of partially inhibitory concentrations of galactose and mannose were less inhibitory than their more inhibitory component. The concentration of dextrose required to reverse the inhibitory activity of such mixtures was not greater than the minimum concentration required to antagonize the action of the more inhibitory component.

The antagonism of galactose inhibition by dextrose is not due to dextrose impeding galactose uptake.

INTRODUCTION

THE present investigation was prompted by the observation that mannose and galactose were, by comparison with other monosaccharides, very inhibitory to the growth of excised tomato roots (Ferguson, Street, and David, 1958*a*) and by the much earlier finding of Knudson (1917), working with

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[Annals of Botany, N.S. Vol. 22, No. 88, Oct. 1958.]

Canadian field pea and wheat plants grown in solution culture, that the toxicity of these sugars was overcome by the simultaneous addition of dextrose to the culture solution.

EXPERIMENTAL

General experimental procedures

These were as described in the previous paper (Ferguson, Street, and David, 1958a).

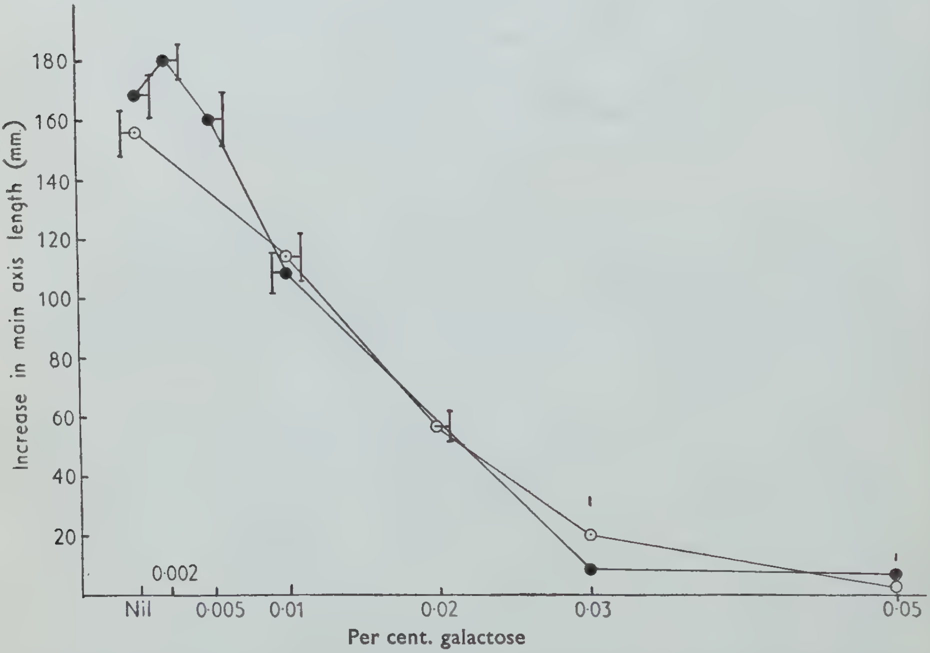

FIG. 1. Inhibition of main axis extension growth resulting from the addition of *d*-galactose to root culture medium containing 1·0 per cent. sucrose. Vertical lines with the mean growth values level with their midpoints have vertical extents = 2 × the standard error of the mean values. Results of two experiments shown.

The inhibition of excised root growth by galactose

d-galactose was added to medium containing FeEDTA as iron source and 1 per cent. sucrose. The sucrose was 'etherized' and then incorporated aseptically with the other medium constituents previously sterilized by autoclaving. The growth inhibition resulting from the galactose additions was the same whether the galactose was sterilized by autoclaving with the other medium constituents, was 'autoclaved separately', or was 'etherized'.

The inhibitory activity of galactose is shown in Fig. 1 where mean values for increase in main axis length (I.M.A.) are plotted against galactose concentration. Galactose at a concentration of 0·01–0·015 per cent. caused 50 per cent. inhibition, and at a concentration of 0·05 per cent. caused complete inhibition of main axis extension.

Roots partially inhibited in growth by galactose were characterized by a paucity of laterals but their main axes were straight and white. At a concentration of 0·05 per cent. the inhibited tips turned brown during the 7-day incubation period. On transfer to standard sucrose (1 per cent.) medium after 3 days' incubation in galactose-containing medium, roots inhibited by 0·04 per cent. galactose resumed main axis growth; roots inhibited by 0·075 per cent. showed only occasional extension of lateral initials.

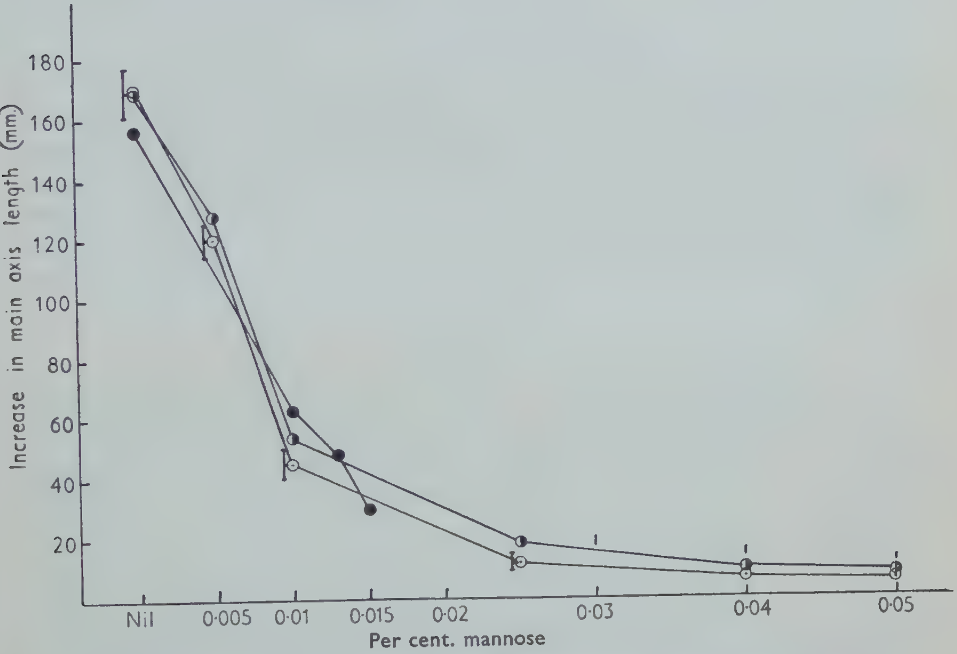

FIG. 2. Inhibition of main axis extension growth resulting from the addition of *d*-mannose to root culture medium containing 1·0 per cent. sucrose. Standard errors shown as in Fig. 1. Results of three experiments shown.

Roots which had developed in standard medium for 3 days showed the full reduction in growth-rate in less than 24 hours when transferred to medium containing 0·03 per cent. galactose. Conversely, root tips inhibited for 3 days by this concentration of galactose and then transferred to standard medium had reached the growth-rate typical of roots in the latter medium within 24 hours. The main axis meristems of roots cultured in medium containing 0·03 per cent. galactose for 4 (7-day) passages were still capable of showing full restoration of their growth activity when placed in standard culture medium.

The inhibition of excised root growth by mannose

The inhibitory activity of *d*-mannose is shown in Fig. 2. Mannose at a concentration of 0·005–0·01 per cent. caused 50 per cent. inhibition of main axis extension. The minimum concentrations of mannose and galactose which caused 90 per cent. or higher inhibition were similar (0·04–0·05 per cent.). Comparing concentrations of mannose and galactose which caused similar degrees of main-axis inhibition, mannose was less inhibitory to lateral development. For instance, 0·01 per cent. mannose and 0·015 per cent. galactose which cause similar degrees of main axis inhibition are contrasted in that with mannose there is approximately a 60 per cent. and with galactose not less than a 90 per cent. reduction in the values for lateral number and total length of laterals per root. Further root tips fully inhibited by mannose did not turn brown during the incubation period.

The survival of meristematic activity in fully inhibitory concentrations of mannose is higher than with galactose. After 7 days' treatment with 0·2 per cent. mannose (4 times the minimum fully inhibitory concentration) more than 50 per cent. of the main axis meristems are able to resume growth when transferred to standard medium and all the roots show lateral development. After 7 days' treatment with 0·075 per cent. galactose (only slightly above the minimum fully inhibitory concentration) main axis meristems are no longer functional and only on some roots is lateral development still possible.

The reversal of galactose and mannose inhibitions by dextrose

Tests of the influence of dextrose additions on the growth inhibitions caused by galactose and mannose were made by autoclaving the inorganic salts (iron supplied as FeEDTA) and vitamins with the inhibitory sugar and then incorporating the sucrose (1 per cent.) and dextrose either by 'autoclaving separately' or by 'etherizing'.

The growth effects of a number of galactose concentrations ranging from 0·05 to 0·4 per cent. were tested in presence of a range of dextrose concentrations. Figs. 3 and 4 show the results obtained for the galactose concentrations 0·05, 0·075, 0·1, 0·15, 0·2, 0·3 per cent. The growth values are expressed as percentages of the control growth in medium containing 1 per cent. sucrose as the only carbohydrate. These results show that the inhibitory effect of galactose concentrations up to 0·1 per cent. can be fully prevented by the simultaneous addition of dextrose and that within this concentration range the minimum fully effective dextrose addition bears an almost fixed relationship to the galactose concentration. The ratio of dextrose:galactose required for reversal of the inhibition is approximately 5:1. With galactose additions above 0·15 per cent., the addition of dextrose only partially reverses the growth inhibition.

The growth effects of a number of mannose concentrations ranging from 0·05 to 0·7 per cent. were tested in presence of added dextrose. Figs. 5 and 6 show the results obtained for the mannose concentrations 0·05, 0·1 (see also Fig. 7), 0·3, 0·4, and 0·5 per cent. The inhibitory effect of mannose concentrations up to 0·4 per cent. could be effectively antagonized by the simultaneous addition of dextrose. The minimum dextrose: mannose ratio for reversal of inhibition was, over this range, approx. 3·5:1. With concentrations of mannose above 0·4 per cent. only partial reversal of the growth inhibition resulted from the dextrose additions.

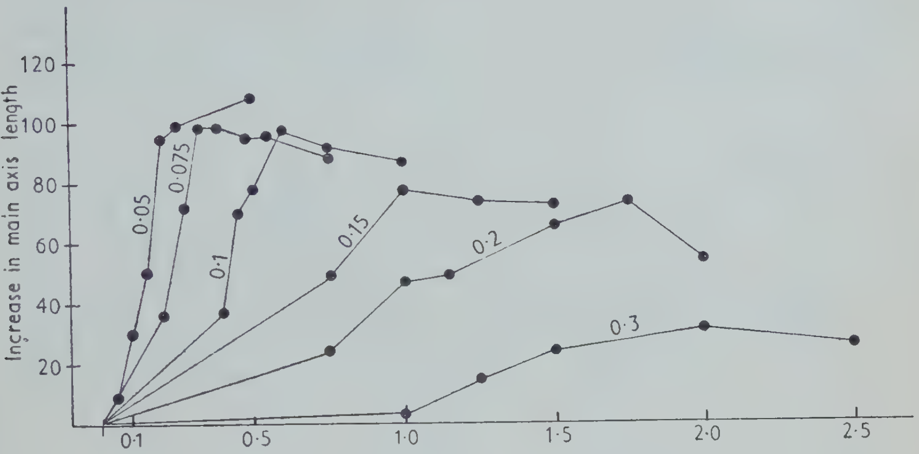

FIG. 3

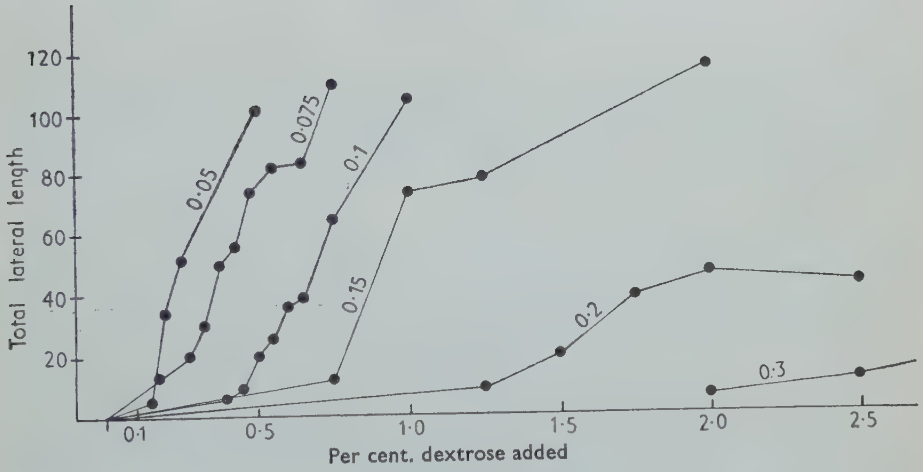

FIG. 4

FIG. 3 and 4. The reversal of the galactose inhibition of excised root growth resulting from dextrose additions. The concentration per cent. of galactose indicated against each graph. Growth values expressed as percentages of the growth values obtained in the control medium (standard medium containing 1·0 per cent. sucrose).

Fig. 3. Values for the mean increase in main axis length.

Fig. 4. Values for the mean total lateral length per root.

Previous growth of roots for 3 days in a medium containing sucrose and dextrose did not decrease the sensitivity of the roots to inhibition by mannose or galactose as compared with a similar period of growth in medium containing only sucrose.

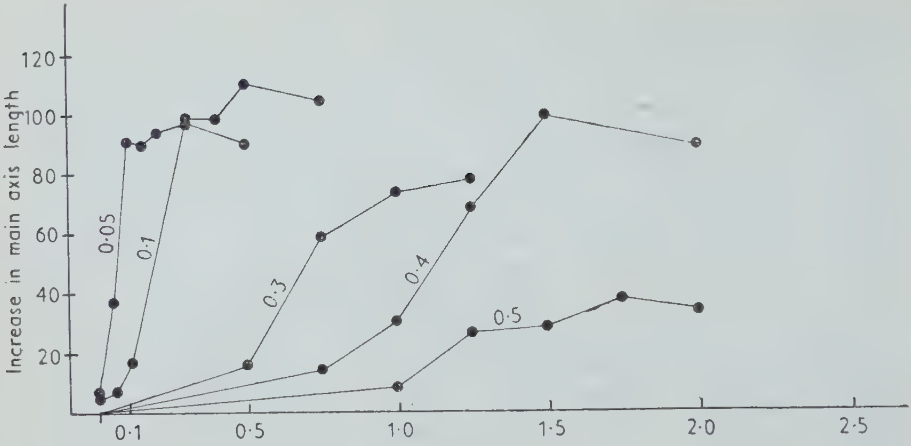

FIG. 5

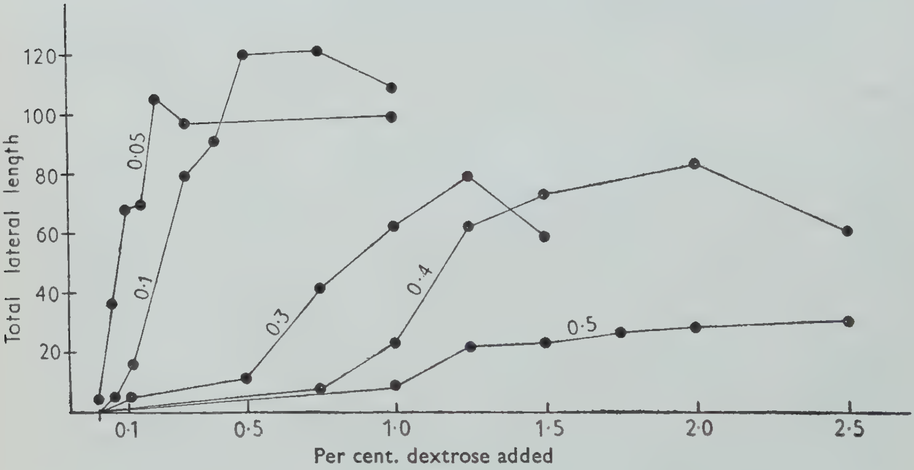

FIG. 6

FIG. 5 and 6. The reversal of the mannose inhibition of excised root growth resulting from dextrose additions. The concentration per cent. of mannose indicated against each graph. Growth values expressed as percentages of the growth values obtained in the control medium (standard medium containing 1 per cent. sucrose).

Fig. 5. Values for the mean increase in main axis length.

Fig. 6. Values for the mean total lateral length per root.

The activity of other sugars as antagonists of the galactose and mannose inhibitions of growth

Knudson (1917) reported that sucrose acted as an antagonist of galactose toxicity in his experiments. The results of our experiments designed to study the effect of the sucrose concentration of the medium on the inhibition of excised root growth by added galactose and mannose have, however, proved difficult to interpret in view of the marked effects of sucrose concentration itself upon growth. With supra-optimal sucrose concentrations (2 and 3 per cent.) partially inhibitory concentrations of galactose and mannose produced less percentage inhibition of linear growth than in presence of 1·5 or 1·0 per cent. sucrose. However, linear growth values were depressed by higher sucrose concentrations so that growth in presence of a supra-optimal sucrose concentration plus mannose or galactose was usually lower and never significantly superior to growth with a more nearly optimal sucrose supply and the same concentration of inhibitory sugar. Furthermore, increase in sucrose concentration did not decrease the minimum concentration of dextrose required to reverse completely galactose or mannose inhibition. There was, therefore, in the present series of experiments, no clear-cut evidence that sucrose counteracted the inhibitory effect of either galactose or mannose.

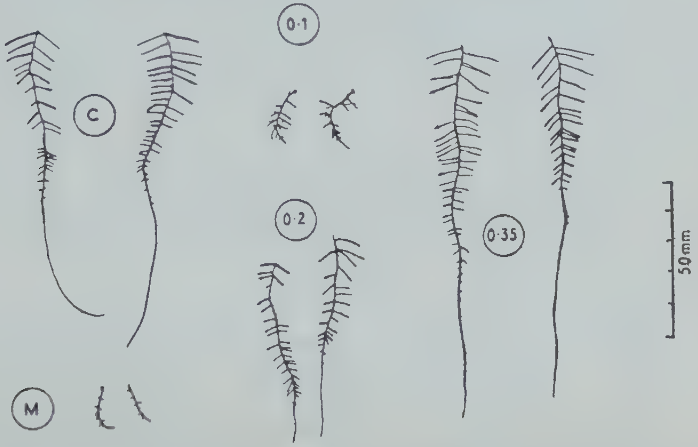

FIG. 7. Reversal by dextrose of the inhibition of excised root growth caused by 0·1 per cent mannose. All roots cultured for 7 days at 27° C. C = control roots from standard (1% sucrose) medium. M = roots from this medium supplemented by 0·1% mannose. 0·1, 0·2, and 0·35 = roots from medium supplemented by 0·1 per cent. mannose but also containing dextrose at the percentage concentrations indicated (0·1, 0·2, and 0·35 per cent.), respectively.

Maltose was, however, found to be a weak antagonist of both galactose and mannose inhibition (Table 1). In these experiments, maltose was sterilized by filtration and therefore hydrolysis prevented during preparation of the medium. The maltose sample used was not examined chromatographically but other samples of the same quality submitted to this test have not shown dextrose contamination. Galactose-inhibited roots showing increased growth as a result of a maltose addition frequently showed a 'kink' in the main axis at the point marking the apex of the initial root meristem (10 mm. from the cut end) suggesting that a portion of the original meristem became active after an initial period of complete inhibition.

D-xylose is quite inhibitory to excised tomato-root growth (Ferguson, Streeta, and David, 1958*a*). Nevertheless, it can be shown to reverse partially the growth inhibition due to mannose and to an even greater extent that due

TABLE 1

The Antagonism of Galactose and Mannose Inhibition of Excised Root Growth by Maltose

Treatment	Replicate No.	Mean growth values*		
		I.M.A.	L.N.	L.L.
Standard medium (1% sucrose)	7	97·3	27·6	125
+0·05% galactose (G)	9	2·1	0	0
+0·05% (G)+1·5% maltose (M)	9	29·8	6·2	11·4
+0·05% G+2·0% M	9	34·8	6·4	18·1
+0·05% mannose (Man.)	9	7·7	3·8	4·3
+0·05% Man+1·0% M	9	18·0	6·8	25·0
+0·05% Man+3·0% M	9	19·8	2·8	10·3

* I.M.A. = increase in main axis length (mm.).
L.N. = number of laterals per root.
L.L. = total lateral length per root (mm.).

TABLE 2

The Antagonism of Galactose and Mannose Inhibition of Excised Root Growth by d-*xylose*

Treatment	Replicate No.	Mean growth values		
		I.M.A.	L.N.	L.L.
Standard medium (1% sucrose)	9	150·8±5·0	43·8	217
+0·05% galactose (G)	9	3·9	0	0
+0·5% xylose (X)	9	8·9	0	0
+0·05% G+0·5% X	9	11·3	0	0
+0·05% G+1·0% X	8	47·6±5·1	0	0
+0·05% G+ 1·5% X	9	72·9±6·9	7·6	22·3
+0·05% G+2·25% X	8	58·6±4·0	3·8	10·6
+0·02% mannose (M)	9	26·2±1·3	9·8	42·2
+0·02% M+0·06% xylose	9	37·4±3·2	10·9	52·2
+0·05% mannose (M)	9	8·0	3·9	4·1
+0·05% M+0·5% X	9	26·1	1·0	3·0

to galactose (Table 2). The relatively low inhibitory activity of mixtures of galactose and xylose containing each sugar at a concentration which singly would completely inhibit root growth indicated a strong mutual antagonism of their respective growth inhibitory activities. However, the inhibitory effects of xylose (used at 0·3 and 0·5 per cent.) were, unlike those of galactose and mannose, not reversible by the simultaneous presence of dextrose when tested at concentrations ranging from 0·1 to 1·5 per cent. Dextrose has also been found to be ineffective as an antagonist of the activity of all other inhibitory monosaccharides (Ferguson, Street, and David, 1958*a*) with the exception of galactose and mannose.

Laevulose, *d*-rhamnose, *d*- and *l*-arabinoses, lactose, and raffinose, when tested over a wide range of concentrations, showed no activity in reversing the inhibitions due to galactose and mannose. Dulcitol did not relieve galactose inhibition, nor mannitol the inhibition due to mannose.

The effects of galactose : mannose mixtures on excised root growth

Table 3 gives the results of specimen determinations of the inhibitory effects of mixtures of galactose and mannose, each sugar being present in the mixture at a concentration which singly would cause only incomplete growth inhibition. These results show that there is no summation of the individual inhibitory activities; the mixtures permitted a level of excised root growth either intermediate between or above the levels occurring in presence of each sugar added separately. In a number of instances, therefore, there occurred a significant reversal of the inhibitory effect of the more inhibitory sugar by the simultaneous presence of an inhibitory concentration of the second sugar.

TABLE 3

The Inhibition of Root Growth by Mixtures of Galactose and Mannose added to Medium Containing 1 per cent. Sucrose

Treatment	Replicate No.	Mean growth values		
		I.M.A.	L.N.	L.L.
Standard medium (1% sucrose)	9	149·3±6·1	48·0	267
+0·02% galactose (G)	9	37·2±2·7	2·2	
+0·01% mannose (M)	9	64·2±5·5	17·8	120
+0·02% G+0·01% M	9	46·7±6·7	3·6	9
+0·0125% M	9	57·4±9·3	18·0	118
+0·02% G+0·0125% M	9	59·8±5·2	9·9	8
+0·015% M	9	29·7±3·9	6·8	31
+0·02% G+0·015% M	9	50·1±3·1	3·1	6
+0·02 mannose (M)	8	22·6±2·5	7·6	31
+0·02% M+0·0125% G	10	35·6±2·5	6·4	32
+0·02% M+0·02% G	9	34·9±1·8	4·4	20
+0·02% galactose (G)	9	40·8±7·0	7·1	19
+0·02% G+0·008% M	9	58·3±3·2	6·8	21
+0·02% G+0·012% M	9	51·3±4·3	6·4	22

Study of the dextrose reversal of the growth inhibition resulting from mixtures of galactose and mannose showed that the minimum effective concentration of dextrose required for maximum reversal was not above that which would have been required to antagonize the effect of the more inhibitory component of the mixture. Fig. 8 gives the results of an experiment involving 0·05 per cent. galactose and 0·1 per cent. mannose added singly and in combination and the effects of adding 0·25, 0·5, and 1·0 per cent. dextrose to these test media. This kind of experiment could not be made sufficiently critical to decide whether the minimum level of dextrose causing maximum reversal of inhibition was intermediate in level between the two minimal concentrations required to antagonize each component separately.

DISCUSSION

Since the pioneer work of Knudson (1915, 1917) a number of workers have reported on the toxicity of galactose and mannose to plant roots. Wachtel (1943), working with cress seedlings found galactose was relatively inactive but that mannose was strongly inhibitory to root growth. Steinberg (1947) noted marked inhibition of tobacco seedling root growth by a number of sugars including mannose and galactose; mannose was exceptional in that it also strongly inhibited shoot growth. Burström (1948) reported galactose to be extremely toxic to seedling wheat roots but to have little or no inhibitory effect (at 0·1 M. concentration) on the growth of roots of *Helianthus annuus* (sunflower) and *Linum usitatissimum* (flax). Stenlid (1957) reported that wheat seedling roots were 50 per cent. inhibited in their growth by 2×10^{-4} M. galactose or by 3×10^{-4} M. mannose.

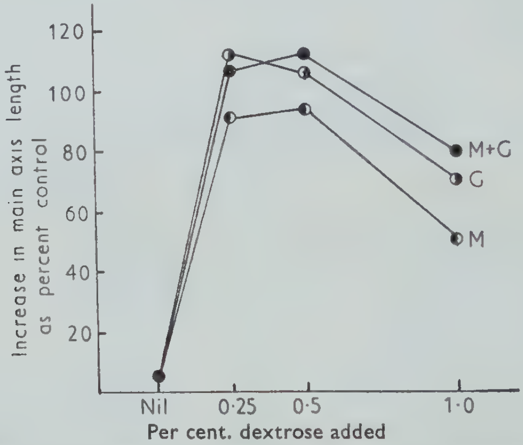

FIG. 8. The reversal of galactose and mannose inhibitions by dextrose additions. Inhibitory sugar additions:

G = 0·05 per cent. galactose.
M = 0·10 per cent. mannose.
M+G = 0·10 per cent. mannose+0·05 per cent. galactose.

Values for mean increase in main axis length expressed as a percentage of this growth value in the control medium (standard medium containing 1·0 per cent. sucrose).

Excised roots seem to be even more sensitive to the toxic action of these sugars. White (1940) reported complete growth inhibition and discoloration of the meristem when galactose was tested as a carbon source for excised tomato roots. David (1954) reported that 0·2 per cent. galactose, when added to standard (2 per cent. sucrose) medium, completely inhibited the growth of excised roots of sunflower, flax, and *Medicago sativa*. Lucerne roots were also strongly inhibited by 0·5 per cent. mannose under these conditions. These findings regarding galactose were confirmed and extended by Frakas (1954) who reported it to be a very active inhibitor of excised root growth in seventeen species.

Detailed comparison of the results reported by these workers emphasizes that the roots of different species differ in their absolute and relative sensitivities to inhibition by galactose and by mannose. The quantitative and qualitative differences in the inhibitory effects of these two sugars to the excised tomato roots used in the present investigation also suggests that they may act in different ways as growth inhibitors. The work of Ordin and Bonner (1957) on the inhibition of *Avena* coleoptile section extension by galactose has shown that this sugar becomes incorporated into the cell wall and may, therefore, act by interfering with the normal progress of cellulose synthesis. Both wheat roots (Burström 1948) and *Avena* coleoptiles (Ordin and Bonner, 1957) appear to be able to respire galactose. By contrast, Stenlid (1954) has shown that mannose strongly depresses the oxygen uptake and chloride absorption of excised seedling wheat roots. The effects of these and other sugars on the respiration of excised tomato roots will be described in detail in a subsequent paper in this series and will serve to emphasize the different actions of these toxic sugars.

The observation that with both galactose and mannose a minimum ratio of dextrose: toxic sugar must be established to achieve maximum reversal of the growth inhibition is not incompatable with the possibility that the antagonism between dextrose and these sugars is competitive. David (1954), in studying the dextrose reversal of galactose and mannose inhibitions of the growth of lucerne roots, has also found that the amount of dextrose required for maximum reversal increases proportionately with increase in concentration of the inhibitory sugar. Michaelis-Menten (1913) enzyme-kinetics enable a test to be devised for the presence of competitive inhibition (Lineweaver and Burk, 1934). This involves plotting of the reciprocal of reaction velocity against that of substrate concentration. Then the presence of a competitive inhibition increases the slope of the resultant line but the intercept (reciprocal of v max) remains constant (at 1·0). Stenlid (1957) has attempted to apply such an analysis in his work with wheat roots. His data was, however, very limited and his conclusion that there is 'a fairly good agreement with theory for *d*-galactose and *d*-mannose' must, in our view, be accepted with caution. For comparison with Stenlid's (1957) data, a double reciprocal plot of data drawn from our experiments involving the reversal by dextrose additions of the galactose inhibition of excised tomato root growth is shown in Fig. 9.

Even if a strictly competitive interaction at an enzyme surface is involved between substrate (dextrose) and inhibitor (mannose or galactose) the data obtained in growth studies with excised roots will only conform to theory if (1) main axis growth is linearly related to the percentage inhibition of the enzyme reaction involved in antagonism and (2) the concentration of dextrose remains constant during the duration of the growth period. The first of these difficulties will be circumvented when the metabolic action of galactose and mannose is better understood. The second condition is not fulfilled by the excised tomato root for dextrose is liberated into the culture medium as a consequence of the utilization of sucrose for growth (Dormer and Street, 1949). It must also be emphasized that a high level of variability occurs in roots grown in slightly inhibitory media, precluding the accurate determination of low degrees of growth inhibition.

These quantitative studies of the dextrose reversal of inhibition together with the mutual antagonism between mannose and galactose do, however, raise the possibility that all three sugars compete for a common site freely exposed to the external solution. This made it important to test the contention of Knudson (1917) that dextrose antagonized the toxicity of galactose by preventing its absorption. Evidence indicating that this is not so is contained in the Appendix to the present paper.

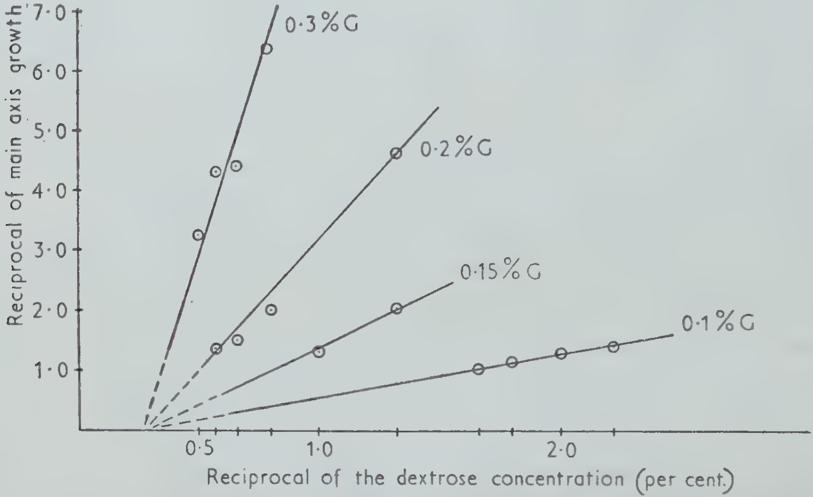

FIG. 9. The reciprocal of growth (percentage of control) plotted against the reciprocal of the concentration of dextrose added. Data from experiments involving the reversal of galactose inhibition by dextrose. The concentration of galactose (G) involved in each test indicated against the resultant line.

For the purpose of discussion it may, therefore, be suggested that dextrose is normally involved at the sites where galactose and mannose disturb metabolism and in consequence act as growth inhibitors. A comparison of the molecular structures of dextrose, *d*-galactose, *d*-mannose, and *d*-xylose suggests that the groups attached to carbon atoms 1, 2, and 4 may be involved in the enzyme-dextrose combinations. The substitution of the aldehydic by a primary alcoholic group at C atom 1 as in dulcitol and mannitol gives molecules which are inactive both as growth inhibitors or as antagonists of galactose or mannose. Galactose by 1:2 attachment and mannose by 1:4 attachment could either give rise to inactive enzyme-substrate compounds thereby blocking a biochemical pathway or could act as substrates in place of dextrose. The activity of xylose as an antagonist of galactose and mannose would then be explained by the correspondence in orientation of the H and OH groups of C2 and C4 in xylose and dextrose, making possible a three-point attachment. The fact that xylose alone is less toxic than either galactose or mannose would require the further postulate that either xylose metabolism is less disruptive or that the xylose-enzyme complex is inactive and that this blocking of metabolism is less deleterious than the disruption caused by the products arising from galactose and mannose metabolism. If two enzyme centres are involved then both these possibilities may be simultaneously operative.

If, following indications from earlier work, it is suggested that galactose is an inhibitor of growth by virtue of its being metabolized, then the presence of dextrose, xylose, and mannose at this point in metabolism must be beneficial either because they serve as alternative metabolites or because they block galactose metabolism. If, by contrast, mannose is an inhibitor because it blocks some biochemical sequence in respiration then this sequence must be activated to different extents by dextrose, xylose, and galactose. Clearly, further progress in our understanding of the phenomena described in this paper depends upon further study of the fate of the absorbed galactose and mannose.

APPENDIX

By S. B. DAVID

The uptake of galactose and dextrose by excised roots of Medicago sativo

1·5 to 2·0 g. fresh weight of 7-day old, washed, excised lucerne roots were placed in 25 ml. sterile root culture medium (minus sucrose) containing initially 0·2 per cent. of one or both of the test sugars, dextrose and galactose. Uptake at 27° C. of these sugars was followed for 96 hr. sampling every 24 hr.

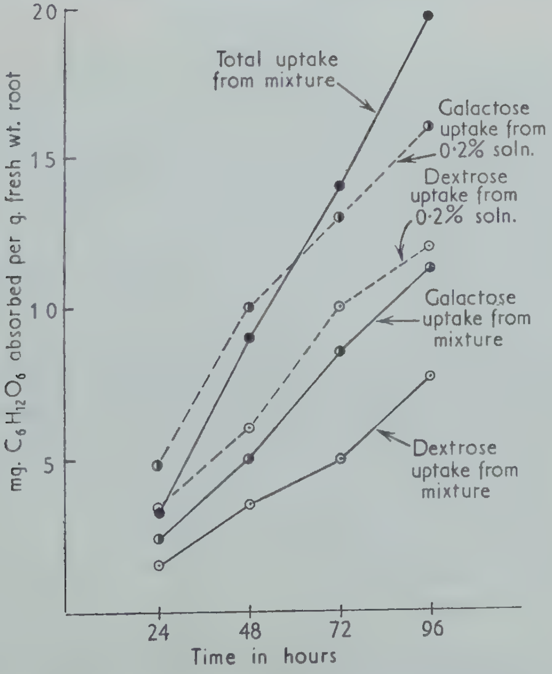

FIG. 10. The uptake of dextrose and galactose from simple solutions (0·2 per cent.) and from a mixed solution (0·2 per cent. of each sugar) by excised lucerne roots.

The reducing sugar content was determined by the method of Somogyi

(1952), with the modification of bubbling nitrogen through the reaction mixture both before and after the heating as suggested by Wager (1954). Extinction coefficients were determined with the Hilger Spekker Absorptiometer (H 760) using a Kodak Filter No. 5 (peak transmission 500 mμ).

The galactose concentration in medium containing dextrose and galactose was determined by fermenting off the dextrose using washed 'DCL' brand baker's yeast in the form of a 20 per cent. suspension as recommended by Greenmann (1950). 0·5 ml. culture medium was incubated with 2 ml. yeast suspension for 30 min. at 33° C. The yeast was removed by filtration and the filtrate adjusted to a suitable volume for determination of its residual reducing sugar content. The yeast suspension was prepared fresh each day.

The results of a typical experiment are shown in Fig. 10. These show that in mixed solution there is no preferential absorption of dextrose or galactose, but that both sugars are actively absorbed in amounts paralleling their rates of uptake from single sugar solutions.

Grateful acknowledgement is made to the Royal Society for a grant to purchase the Hilger Spekker Absorptiometer.

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Effect of Gibberellic Acid on Rate of Extension and Maturation of Pea Internodes

BY

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ABSTRACT

Gibberellic acid (GA) does not delay maturation of pea internodes; on the contrary, maturation (i.e. cessation of extension) takes place slightly earlier. Thus the increased length of internodes resulting from GA treatment is due entirely to increased rate of extension.

In this experiment, GA treatment of plants accelerated the visible production of the first flower bud by about 4 days: the node bearing the first flower was not altered. The total number of flower buds produced by the end of the experiment was increased as a result of GA treatment, but many of those first formed on plants receiving high doses (1–10μg.) withered before opening.

INTRODUCTION

INTERNODE extension in dwarf peas is limited by internal factors. Substitution of a single gene will enable much greater extension to take place and a qualitatively and quantitatively similar increase in internode length results from treatment of dwarf seedlings with gibberellic acid (GA). Thus GA has been considered to remove limitations on growth (Stowe and Yamaki, 1957; Brian and Hemming, 1958). GA increases the rate of extension of pea internodes (Brian and Hemming, 1955) and this could by itself increase internode length. But internode extension is also normally limited by processes of maturation, apparently acting by altering the physical properties of cell-walls, which finally prevent further cell extension. As a consequence, in a pea seedling usually only two visible internodes are extending at any moment, all internodes below these having ceased extension. Increase in internode length and final plant height could also result from a delay in this process of maturation. The experiment described below was carried out to determine whether GA has any effect on maturation of internodes of intact plants. Some observations on flowering made during this experiment are also briefly described.

MATERIALS AND METHODS

'Meteor' pea seedlings were given doses of 0·01, 0·1, 1, and 10 μg. gibberellic acid as previously described (Brian and Hemming, 1955). At the time of first treatment internode 4 was the first uppermost visible internode. Sixteen replicates were used for each treatment. The plants were grown in a 20° C. waterbath in a greenhouse. All internodes above the first true leaf were measured at 48-hour intervals. The experiment was carried on for 30 days,

[Annals of Botany, N.S. Vol. 22 No. 88, Oct. 1958.]

at which time all plants were still growing, though the growth-rate was beginning to decline in all.

RESULTS AND DISCUSSION

Vegetative growth

Mean lengths of internodes at the end of the experiment are shown in Table 1. The two higher doses caused small increases in internode number; from previous experience we should expect the number of internodes in all plants to be similar by the time growth ceases (Brian, 1957). The effects on internode length are comparable with those previously reported (Brian and Hemming, 1955). The mean number of days after the beginning of the experiment required for each internode to reach maturity, i.e. to cease extension, is shown in Table 2.

TABLE 1

Mean Length (mm.) of Internodes at End of Experiment

Dose of GA (μg.)	Internode no.														Total length
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
0	10	19	19	18	21	26	28	24	12	2*	1*	—	—	—	180
0·01	12	21	20	17	22	29	30	23	11*	3*	1*	—	—	—	189
0·1	20	43	31	21	24	30	27	24	11*	2*	1*	—	—	—	234
1·0	24	68	76	67	71	62	41	30	22	14*	8*	2*	—	—	485
10·0	24	74	87	87	94	102	99	89	75	56	42	26*	10*	2*	867

* Immature internodes, still extending at end of experiment.

TABLE 2

Mean Number of Days after Beginning of Experiment when Each Internode Ceased Extension

Dose of GA (μg.)	Internode no.										
	4	5	6	7	8	9	10	11	12	13	14
0	6	10	14	17	20	22	26	28	—	—	—
0·01	6	10	14	17	20	23	26	28	—	—	—
0·1	5	8	12	15	17	21	23	26	—	—	—
1·0	5	8	11	13	16	18	21	23	28	—	—
10·0	5	8	11	13	15	17	19	22	23	26	28

So far from delaying maturity of internodes, GA accelerates the process of maturation. Thus, for instance, internode 10 ceased extension 26 days after the beginning of the experiment in untreated plants, after 19 days in plants treated with 10 μg. GA. This acceleration in maturation is also reflected in the fact that untreated plants had 11 mature internodes at the end of the experiment as compared with 14 in plants treated with 10 μg. GA. However, these data exaggerate the effect, since internodes in GA-treated plants not only finished extension earlier than corresponding internodes in untreated plants, but also commenced extension earlier. The mean time during which internodes 5–11 (i.e. those internodes which matured in all plants, internode 4 being excluded as it had commenced extension before the experiment was begun) were visibly extending is given in Table 3. The GA in doses of 0·1–10μg. significantly reduced the total extension time in every case. Thus the increase in internode length resulting from GA treatment is entirely due to an increased rate of extension, and this has a pronounced effect in spite of more rapid maturation of internode cells.

TABLE 3

Mean Number of Days during which each Internode was Visibly Extending

Dose of GA (μg.)	Internode No. 5	6	7	8	9	10	11
0	6·8	7·0	6·4	6·6	6·6	6·6	6·4
0·01	7·0	6·5	6·1	6·2	7·0	6·5	6·7
0·1	5·8	5·9	5·0	5·3	5·9	5·4	5·4
1·0	5·8	5·4	4·5	5·1	5·2	4·6	4·6
10·0	6·0	5·3	4·8	5·1	5·7	5·0	4·9
Significant difference ($P = 0·05$)	0·7	0·8	0·8	0·7	0·6	0·7	0·7
” ” ($P = 0·01$)	0·9	1·0	1·0	1·0	0·9	1·0	1·0

TABLE 4

Effects of GA Treatment on Flowering

(Data in all but first column are totals for 16 plants at end of the experiment.)

Dose of GA (μg.)	Days to first flower (mean)	Number of flowers: Unopened buds	Withered buds	Opened flowers	Fertilized flowers	Total
0	20·4	2	10	6	37	55
0·01	20·5	—	14	3	34	51
0·1	19·0	—	15	3	35	53
1·0	17·3*	—	29	17	32	78
10·0	16·6*	9	82	20	2	113

*Withered flower bud.

Flowering

Some data on flowering are presented in Table 4. Treatment with GA resulted in slightly earlier appearance of the first flower. It did not affect the node at which the first flower bud was produced, this being at node 9 or 10 in every case. Flowering was hastened to about the same extent as maturity of the internodes in the vicinity. GA treatment also increased the number of flower buds produced by the end of the experiment; however, many of the buds produced on GA-treated plants had very thin peduncles and the buds withered before opening. The number of withered buds increased with the size of the dose of GA. All the buds first produced on plants receiving

1 and 10μg. doses withered in this way, but normal flowers were formed later at higher nodes.

ACKNOWLEDGEMENTS

We are indebted to Mr. J. H. Petty and Mr. P. T. Richmond for assistance with the measurements.

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Techniques for Studying Opaline Silica in Grass Leaves

BY

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With Plates 1 and 2

ABSTRACT

In making surface preparations and sections of grass leaves, special precautions are necessary to recognize and locate silica secreted as opal in epidermal cells. Treatment with ammonia, oxidation with hydrogen peroxide (and, for surface preparations, with chromic acid), and mounting in media of Refractive Index 1·51–1·54 are recommended. Since grass leaves de-silicified by hydrofluoric acid yield consistently good sections, the difficulty of obtaining undamaged opal-bearing sections must be due to the blade encountering opals. Study of sizes and dispositions of opals in surface preparations indicates optimum microtome settings for reducing the frequency of such encounters. In media chosen to make the opals conspicuous it is difficult to discern the surrounding structures, but in most cases phase-contrast or polarization methods overcome this difficulty.

INTRODUCTION

IT has long been recognized that some leaf epidermal cells of the Gramineae tend to accumulate silica. As this is in the solid hydrated form, $SiO_2 . nH_2O$, known to mineralogists as 'opal', these secretions may be conveniently referred to as 'opaline silica' or 'opals'. They have been investigated by various continental botanists, e.g. Grob (1896) and Prat (1936), as part of their intensive studies on epidermal tissues. Such studies are becoming increasingly important in that the shapes of the opals and their accompanying epidermal pattern may help in establishing a more natural grouping of the genera and tribes of the Gramineae (Hubbard, 1948). Furthermore, soil scientists are taking renewed interest in plant opals (the phytoliths of the Russian pedologists) which have long been known to occur in soils (Ruprecht, 1866; Tyurin, 1937). Smithson (1956*b*, 1958) has shown that the opals in the dominant grasses of some North Wales pastures agree in sizes, shapes, and physical properties with phytoliths in the soil.

Many ways of studying plant silica have been described by Netolitsky (1929), most of them involving the 'ashing' of the specimen. In recent work (Parry and Smithson, 1957) it has been found that, although it is often difficult to detect opals in an ordinary botanical preparation, any which are present become very conspicuous if the material surrounding them can be made colourless and sufficiently transparent. Techniques for producing this effect in leaf blades by the use of appropriate chemical reagents and mounting media are described here.

[Annals of Botany, N.S. Vol. 22, No. 88, Oct. 1958.]

Success both in making and interpreting the preparations demands a knowledge of the properties of opal. It is chemically stable and remains when the organic matter of the leaf is destroyed by burning or by heating with concentrated chromic acid, but it is soluble in hydrofluoric acid. The density (2·1–2·2) can be verified if the particles isolated from the leaf are subjected to a micro-flotation technique (Smithson, 1956*a*). The refractive index (R.I.) is low (*c.* 1·42) and consequently the opal has distinctly higher relief than the tissue when preparations are mounted in such media as canada balsam (R.I. *c.* 1·54 or slightly lower if applied in xylene), Gurr's neutral mounting medium (*c.* 1·51), and cedarwood oil (*c.* 1·51).

As the opals are non-sectile objects, microtome work on each species has been preceded by a study of preparations which do not involve sectioning, particularly of leaf segments and surface preparations. These have indicated the degree of probability of making microtome cuts successfully, i.e. without striking an opal and so mutilating the section. The proportion of successes to be expected varies from specimen to specimen according to species, maturity of leaf, direction and thickness of section, &c., and is often very low. The chance of success may even be zero for any but an absurdly thick section, particularly for transverse sections in species which contain rod-like opals oriented longitudinally. Even if only a few successes have resulted from a large number of attempts the information obtained has usually justified the effort. Blades from which the opal has been chemically removed have also been sectioned so as to study structures which survive this treatment. By combining the facts learned from the different techniques it is possible to visualize in a three-dimensional sense the positions of the opals within the structure of the leaf.

SURFACE PREPARATIONS OF THE EPIDERMIS

Methods of isolating small fragments of the lower epidermis by scraping and peeling as described by Prat (1948) were satisfactory for some species but failed to isolate the upper from the lower epidermis, especially for leaves with heavily ribbed surfaces. A more satisfactory method was to warm the blades in dilute ammonia (880 ammonia, 1 vol.; water, 3 vol.), leave immersed for 24 hours and, after careful washing with water, to treat with hydrogen peroxide (6 per cent. or '20 volumes') for 1 to 6 days according to the species and the maturity of the leaf. For the very rigid and well-developed blades of *Sieglingia decumbens* 3 days in hydrogen peroxide was adequate, whereas for the blades of *Phyllostachys nigra* and allied species a 6-day treatment did not prove excessive.

The blades were then dehydrated up to 95 per cent alcohol. After this they were divided into lengths of 3–5 mm., each length also having one of its longitudinal edges cut away. The lengths were then cleared in cedarwood oil, the clearing from alcohol being made very gradually, the final clearing lasting at least 24 hours (Peacock, 1940). Cedarwood oil was found to be an ideal medium for rendering the tissues less conspicuous than the opals. Less damaging than other media, it is also suitable as a temporary mountant (Plate 1, Fig. 1). Examined in this condition the upper and lower epidermis are seen at different levels of focus in the same field. While still in cedarwood oil it is possible for one epidermis to be separated from the other by careful manipulation with a fine scalpel or needle, and, despite some inevitable tearing, to end up with them lying in one plane and joined by the uncut edge of the leaf (Plate 1, Fig. 2).

The choice of the permanent mounting medium is very important for these epidermal preparations. For example, Gurr's neutral mounting medium ensures an excellent view of the opals whilst the remainder of the tissues are rendered less prominent but not completely obliterated (Plate 1, Fig. 3). In Euparol (R.I. *c.* 1·48) the opals are much more difficult to recognize (Fig. 4).

The most transparent surface preparations were obtained by taking material already decolourized by treatment with ammonia and hydrogen peroxide and treating it as follows. We use dilute chromic acid made by diluting with two volumes of water one volume of stock chromic acid (potassium dichromate, 30 g.; water, 250 ml.; concentrated sulphuric acid, 200 ml.). (The addition of 4 ml. concentrated nitric acid to 96 ml. of the dilute chromic acid apparently helps to prevent charring.) A single portion of a decolourized leaf is placed in a dish of this dilute acid, and the specimen is given the operator's undivided attention until the chemical attack has been halted. The dish is placed on a metal plate heated at one corner. A green coloration, due to the reduction of chromic acid, and a slow evolution of bubbles on and in the leaf indicate when the reaction is proceeding satisfactorily, but if the bubbles form rapidly, the dish must be moved to a cooler part of the plate. To ensure that both surfaces are wetted and acted upon by the acid, various devices have been tried (e.g. placing over the specimen a perforated Pyrex watch-glass, convex side upwards so that bubbles escape through the aperture; or pressing the leaf down with a glass rod having one end turned and flattened to form a foot), but, whatever device is employed, repeated attempts may be necessary before good results are obtained. When the specimen has lost its green coloration, the acid is repeatedly diluted with distilled water and siphoned off as the specimen floats. A microscope cover-glass is then coated with a dilute solution of natural canada balsam in benzol, and when the solvent has evaporated, the glass with its 'tacky' surface upwards is put into the liquid and raised with the specimen adhering centrally to it. When the cover-glass and specimen have been dried by gentle warming, undiluted natural canada balsam is added and baked slowly until a sample of the balsam taken on the tip of a needle and cooled in water is found to be slightly brittle when pressed upon glass. The slip is then carefully lowered on to a warm slide coated with similarly baked balsam. If chemical attack has been incomplete, the heat during mounting chars and often spoils the preparation, though sometimes it produces an excellent anatomical picture (Plate 1, Fig. 5).

With the chromic-acid technique, the upper and lower surfaces tend to separate and will float apart if both edges of the leaf have previously been cut.

Alternatively, by cutting only one edge it is possible to end up with the specimen opened out, hinged by the remaining edge. This opening-out may occur during the reaction or the dilution process, or may be brought about when one epidermis has adhered to the cover-slip and the other is free to be turned over by a flow of water obtained by gently squeezing a polythene wash-bottle.

Preparations made by the use of chromic acid are shown in Plate 1, Figs. 6–12, and Plate 2, Figs. 13 and 14, some of them as seen when using the special techniques described under 'Microscopical Examination'.

PREPARATION OF SECTIONS

Short lengths of blades pre-treated with ammonia and hydrogen peroxide and stored in cedarwood oil were embedded in paraffin wax (melting-point 52° C.). For sectioning a 1-day period in ammonia and a 1–2-day period in hydrogen peroxide were adequate for most species. Optimum times may vary within the species, however, according to the degree of maturity of the blades; but with very much longer periods the material becomes partly disintegrated during the subsequent embedding and cutting processes.

Wax impregnation was allowed to proceed slowly and was enhanced by passing the material quickly through xylene from cedarwood oil. Prolonged periods in xylene renders the material very brittle for subsequent handling and sectioning. The lengths were allowed to remain in molten wax for at least 6 days. With shorter periods impregnation was incomplete and sections were badly torn as a result. The impregnation period of 6 days had the effect of badly distorting the blade lengths, and it proved a real difficulty to cut a series of true right-angle longitudinal and transverse sections. The simplest and the most effective way of overcoming this difficulty was to isolate portions of the blade between the mid-rib and the extreme edge of the blade. In a few cases only was the isolated mid-rib successfully sectioned longitudinally.

The frequent tearing of sections was attributed to the fact that when cutting material with a high opal content, the knife would strike many of the opals and drive them through the tissue. By examining surface preparations and observing the sizes and positions of the opals it was possible to decide what thickness of section was likely to give the greatest proportion of successes. For instance, in a sample of *Sieglingia decumbens* it was found that few of the dumb-bell-shaped opals were more than 10 μ in length. A microtome setting of 12 μ was therefore tried and this produced a high proportion of successes (Plate 2, Figs. 15–19). Where larger opals were known to be present, sections of up to 80 μ were cut on a sledge microtome for the study of these bodies.

That the tearing of many sections even at the optimal thickness was due to the presence of the opals was confirmed by the fact that when the material had been pre-treated for 1 hour with concentrated hydrofluoric acid in wax or polythene vessels no tearing resulted (Plate 2, Figs. 20, 21). In all cases, serial sections were more easily obtained by allowing the paraffin blocks, with the cut surface of the material exposed, to stand in distilled water for 24 hours before microtoming (Johansen, 1940). Ribboned sections were fixed to the glass slides by means of Haupt's adhesive, and after the removal of the paraffin wax by means of xylene some of the slides were made permanent with Gurr's neutral mounting medium.

MICROSCOPICAL EXAMINATION

When investigating the locations of opals within the leaf one meets the difficulty that the techniques for making their presence evident do so by making the surrounding tissue almost invisible (Plate 1, Figs. 6, 12; Plate 2, Fig. 13), while techniques for emphasizing the tissues obscure many of the opals (e.g. Fig. 4). If, however, the microscope is fitted with phase-contrast or polarizing equipment (or, preferably, with both) the tissues may be made to exhibit good contrast in preparations in which this is impossible with the ordinary microscope. Phase-contrast examination may be done by the standard methods, but since birefringent materials are being examined it is preferable to insert a substage polar and to rotate it until maximum contrast is obtained Plate 1, Fig. 7; Plate 2, Fig. 17).

On the polarizing microscope the two polars are generally set in the E.–W. and N.–S. positions, in which case for surface preparations and transverse sections the leaf axis should lie NW.–SE. or NE.–SW., thus giving maximum contrast and showing polarization colours occasionally reaching the second order of Newton's scale (see below). Under these conditions, however, only fibres of sclerenchyma show much improvement on the image obtained with ordinary light (Plate 2, Figs. 13, 14), since feebly birefringent objects such as long cells may show little more than grey, whilst opals, being isotropic, may be lost in the darkness of the field (Plate 1, Fig. 8). If, instead of leaving the polars crossed at precisely 90°, one of them is turned some 5° or 10°, the field becomes grey and opals are visible (Figs. 9, 19). The greatest improvement, however, is obtained by inserting a quarter-wave mica plate (see page 548) in the slot of the polarizing microscope (Figs. 10, 11, 18). With this set-up, objects which have appeared grey may be changed at will to black or white simply by changing their orientation with respect to the mica plate, so that although in one position the long cells are white and the guard cells of the stomata are dark, a rotation of the microscope stage by 90° causes a reversal of these tones (Figs. 10, 11). In both positions the opals are black-edged because their refractive index is very different from that of the medium and they are grey-centred because they are istropic and must show the field tone.

Newton's Scale: The sequence of colours which Newton observed in the air film produced when glass surfaces are pressed together may be produced in many ways, the colours of thin oil films being perhaps the most familiar. Under the polarizing microscope they are produced by birefringent materials and may be utilized both for identifying substances and for producing contrast. When viewed between crossed polars—either nicol prisms or sheets of polaroid—certain structures of the leaf preparations described here show one or more of the following sequence of colours, depending upon the optical properties, thickness, and orientation of the material.

First Order of Newton's Scale	Black	0
	Grey	$\frac{1}{4}$
	White	$\frac{1}{2}$
	Yellow	$\frac{3}{4}$
	Red	1
Second Order (part only)	Blue	$1\frac{1}{4}$
	Green	$1\frac{1}{2}$

Quarter-wave Mica Plate: Sheets of appropriate minerals suitably placed between the polars cause blank areas of the field to assume one of the colours of Newton's scale and at the same time modify the polarization colours of the object. A plate which changes the field colour to red of the first order will in this way produce striking colour contrasts in a botanical preparation, but perhaps the most useful is the quarter-wave mica plate. This consists of a piece of mica cleaved to give first order grey, usually mounted between glass strips and marked to show the vibration directions of the fast and slow rays, one of these being made to correspond to the length of the glass strip, the other being at right angles to it. If an object is showing grey (position $\frac{1}{4}$ on Newton's scale) and a $\frac{1}{4}$-wave plate is introduced, the object may change to white ($\frac{1}{4}+\frac{1}{4} = \frac{1}{2}$). When this happens the effects of the object and the plate are evidently additive, the fast and slow vibration directions of the object coinciding with those of the plate. Hence, if the object is rotated through 90° the result should be subtractive ($\frac{1}{4}-\frac{1}{4}$), giving zero on the scale, i.e. black.

When the sole purpose is to produce contrast, the mica plate need not be precisely 'quarter wave' or mounted and inserted as in a standard polarizing microscope. Indeed, a selection of unmounted pieces of split mica giving various shades of grey between crossed polars and cut into oriented strips are in many ways preferable, since for each object examined one may select the piece which gives the best contrast, and if the microscope has no slot for inserting a plate the mica may rest on the slide even when using high-power objectives.

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EXPLANATION OF PLATES 1 AND 2

PHOTOMICROGRAPHS OF PREPARATIONS OF GRASS LEAVES

N.B. Although the leaves in Figs. 8–11, 14, 18, and 19 are in the conventional position it was necessary to turn the object through 45° to obtain the contrast depicted in the illustrations.

Surface Preparations (Treatment: Ammonia, hydrogen peroxide)

FIG. 1. *Chusquea culeou*, in cedarwood oil, showing opals in the motor cells. ×85.

FIG. 2. *Molinia caerulea*, in Gurr's neutral mounting medium. Left: Lower epidermis; Right: Upper epidermis. ×85.

FIG. 3. *Sieglingia decumbens*, in Gurr's neutral mounting medium. Lower epidermis. Note the line of conspicuous dumb-bell opals. ×165.

FIG. 4. *Sieglingia decumbens*, in Euparol. Lower epidermis. Dumb-bell opals present but inconspicuous. ×165.

Surface Preparations (Treatment: Ammonia, hydrogen peroxide, dilute chromic acid. All in canada balsam)

FIG. 5. *Sieglingia decumbens*. Lower epidermis. Charred. ×120.

FIG. 6. *Sieglingia decumbens*. Lower epidermis. A line of dumb-bell opals along a rib and smaller scattered opals in short cells. ×165.

FIG. 7. Same field as Fig. 6. Phase-contrast with one polar. ×165.

FIG. 8. Same field as Fig. 6. Polars crossed at 90°. ×165.

FIG. 9. Part of the same field as Fig. 6. Polars crossed at 85°. ×320.

FIG. 10. Same field as Fig. 6. Polars crossed at 90° with quarter-wave mica plate. ×165.

FIG. 11. Part of the same field as Fig. 6, with set-up as in Fig. 10, but with mica plate rotated 90°. ×620.

FIG. 12. *Sieglingia decumbens*. Dark ground illumination. Lines of dumb-bell opals and two fortuitously silicified long cells. ×165.

FIG. 13. *Anthoxanthum odoratum*. Rod-like opals. ×165.

FIG. 14. Same field as Fig. 13. Polars crossed at 90°, showing the fibres of sclerenchyma which overlie the opals.

Sections (Treatment: Ammonia, hydrogen peroxide; Gurr's neutral mounting medium)

FIG. 15. *Sieglingia decumbens*, T.S. End-on view of three dumb-bell opals in the lower epidermis. ×320.

FIG. 16. *Sieglingia decumbens*, L.S. Two of the small opals associated with the long cells of the lower epidermis. ×320.

FIG. 17. Same field as Fig. 16. Phase contrast. ×320.

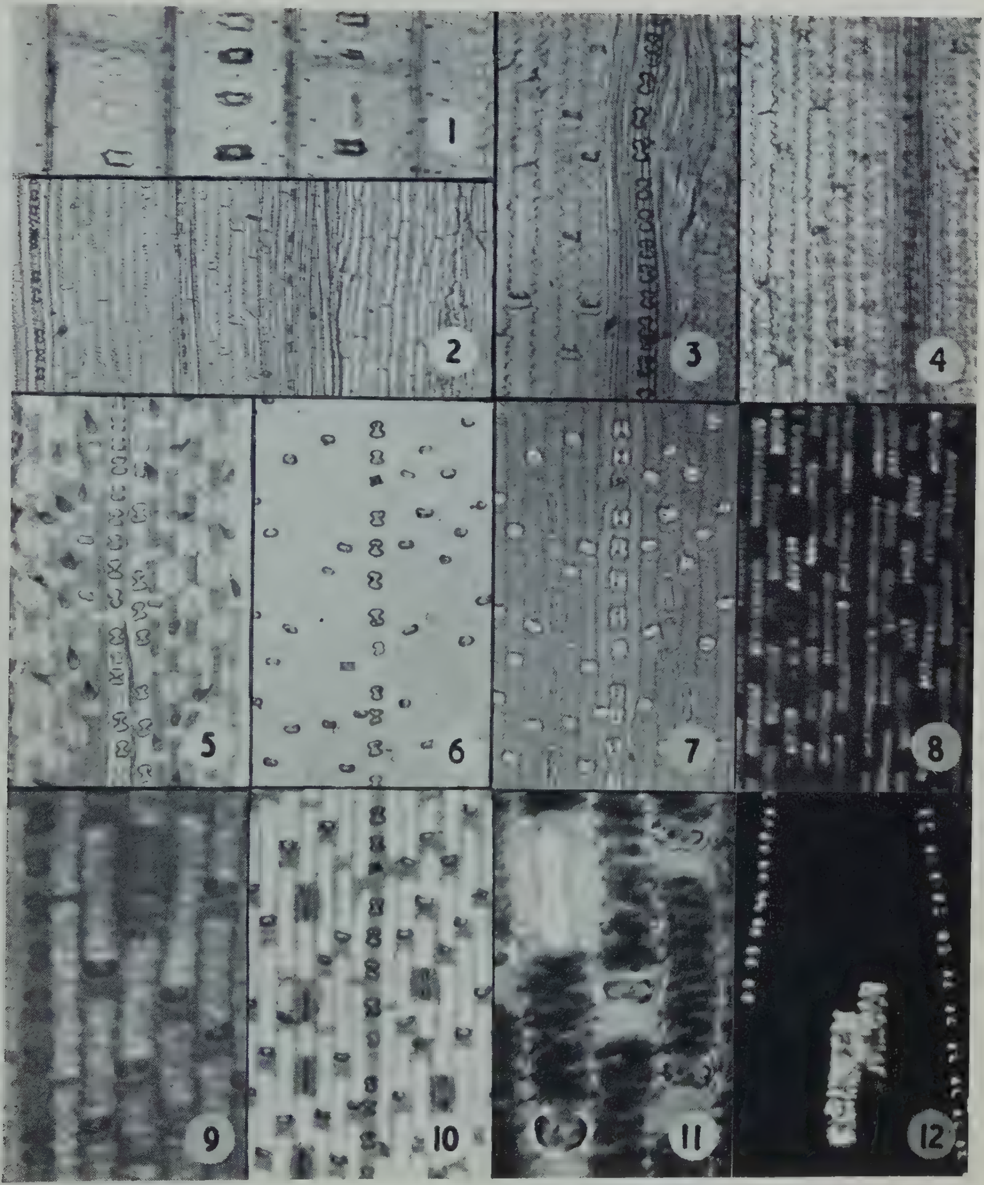
FIG. 18. Same field as Fig. 16 with set-up as in Fig. 10. ×320.

FIG. 19. *Sieglingia decumbens*, L.S. Set-up as in Fig. 9. A line of dumb-bell opals and schlerenchyma (white). ×165.

Sections (Treatment: Hydrofluoric acid; Gurr's neutral mounting medium)

FIG. 20. *Molinia caerulea*, T.S. ×320.

FIG. 21. *Molinia caerulea*, L.S. ×320.



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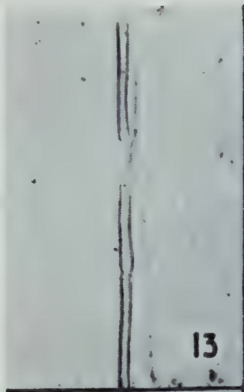
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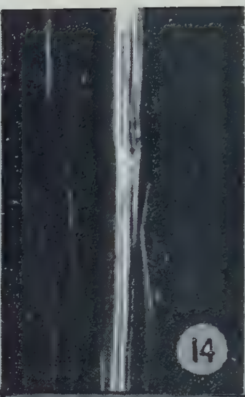
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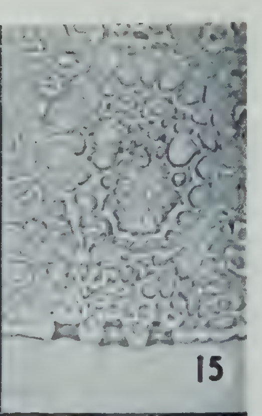
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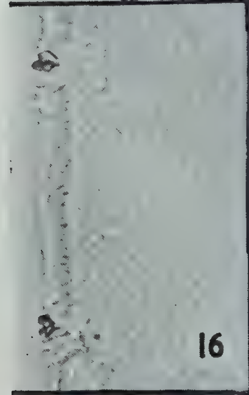
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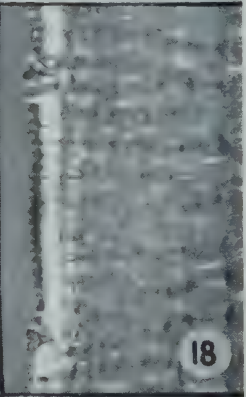
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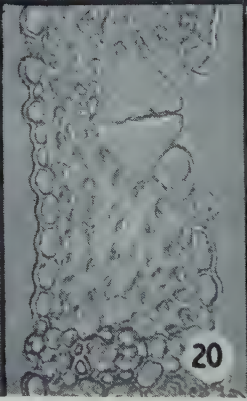
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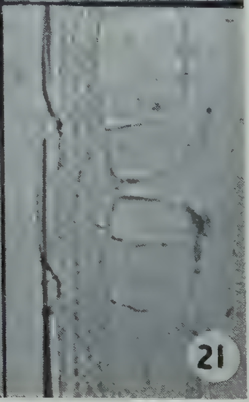
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